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Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte

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SUMMARY

Cortical granules exocytose after the fusion of egg and sperm in most animals, and their contents function in the block to polyspermy by creating an impenetrable extracellular matrix. Cortical granules are synthesized throughout oogenesis and translocate en masse to the cell surface during meiosis where they remain until fertilization. As the mature oocyte is approximately 125 μm in diameter (*Lytechinus variegatus*), many of the cortical granules translocate upwards of 60 μm to reach the cortex within a 4 hour time window. We have investigated the mechanism of this coordinated vesicular translocation event. Although the stimulus to reinitiate meiosis in sea urchin oocytes is not known, we found many different ways to reversibly inhibit germinal vesicle breakdown, and used these findings to discover that meiotic maturation and cortical granule translocation are inseparable. We also learned that cortical granule translocation requires association with microfilaments but not microtubules. It is clear from endocytosis assays that microfilament motors are functional prior to meiosis, even though cortical granules do not use them. However, just after GVBD, cortical granules attach to microfilaments and translocate to the cell surface. This latter conclusion is based on organelle stratification within the oocyte followed by positional quantitation of the cortical granules. We conclude from these studies that maturation promoting factor (MPF) activation stimulates vesicle association with microfilaments, and is a key regulatory step in the coordinated translocation of cortical granules to the egg cortex.

Key words: Meiosis, Cortical granule, Microfilaments, Endocytosis, cAMP, Oocyte maturation, Sea urchin

INTRODUCTION

Eggs of most animal species are fertilized by a single sperm and fusion of these two cells is essential for the activation of the developmental program of the zygote. Cases in which multiple sperm fertilize an egg, a condition known as polyspermy, the supernumerary paternal centrioles and chromosomes usually create aberrant cell divisions that result in death. Thus, eggs have evolved mechanisms to prevent polyspermy. The most conserved mechanism is a permanent modification of the extracellular matrix of the egg to separate physically and biochemically extraneous sperm from the egg. This change at the cell surface results from secretion of dense core vesicles called cortical granules, which are poised in the egg cortex prior to fertilization.

The contents of cortical granules in frogs and sea urchins have been well studied and are found to contain a diverse repertoire of molecules that includes enzymes, structural proteins and glycosaminoglycans (Wessel et al., 2001). Once secreted, these molecules mix with the nascent vitelline layer of the egg and lift it from the cell surface to form a proteinaceous envelope. In mammals, the contents of cortical granules are not as well understood, but they too modify the oocyte extracellular matrix, the zona pellucida, making it non-receptive to subsequent sperm. In general, cortical granules are synthesized throughout oogenesis, accumulating linearly, and are dispersed evenly throughout the cytoplasm of the cell. However, just prior to the resumption of meiosis, apparent by germinal vesicle (oocyte nucleus) breakdown, cortical granules translocate en masse to the cell surface where they dock to the plasma membrane (Berg and Wessel, 1997).

While the stimulus for cortical granule translocation to the plasma membrane is not known in any animal, in sea urchins it does occur temporally coincident with the resumption of meiosis. A general indication of meiotic maturation is movement of the germinal vesicle to the oocyte surface, where it breaks down, and progresses through two rounds of meiotic reduction, resulting in two polar bodies and a haploid complement of chromosomes. Oocytes of most animals are halted at some point in meiosis (MII for frog and mouse, for example) and fertilization reinitiates the meiotic process and the exocytosis of cortical granules. In starfish, a close relative of sea urchins, oocytes are shed with an intact germinal vesicle and 1-methyladenine synthesized in the follicle cells reinitiates meiosis. These oocytes are usually fertilized in MII, and then complete meiosis. If these oocytes are not fertilized within 20

hours after completion of meiosis, they will die by apoptosis (Sasaki and Chiba, 2001; Yuce and Sadler, 2001). By contrast, sea urchin oocytes complete meiosis in the ovary, and the haploid eggs are stored for prolonged periods in the ovarian lumen until induced to shed. Curiously, in sea urchins the cortical granules translocate to the cortex during meiotic maturation, yet they remain attached to the plasma membrane for days, or experimentally for months, without precocious secretion. Within seconds of insemination though, the entire population of cortical granules is secreted.

The process of meiotic maturation in sea urchins is recapitulated in vitro, although the stimulus to re-enter meiotic maturation is not known. Currently, to study changes at meiosis we rely on the spontaneous reactivation of meiosis in isolated oocytes (Berg and Wessel, 1997; Grainger et al., 1986; Harvey, 1956). The resumption of meiotic progression in this oocyte is indicated by the displacement of the germinal vesicle to the future animal pole of the cell. By this time, ~40% of the cortical granules will have already translocated to the cortex (Berg and Wessel, 1997). Thus, cortical granule translocation is an early indicator of meiotic resumption, or may even precede the stimulus for meiosis in this oocyte. As the oocyte is approximately 125 μm in diameter, this means that many of the cortical granules must migrate upwards of 60 μm, through an organelle-laden cytoplasm, to reach the cortex. We have identified several major features of cortical granule translocation. First, we characterized potential biochemical mechanisms used by sea urchin oocytes to initiate meiotic maturation. We were then able to learn that meiotic maturation and cortical granule translocation are inseparable, suggesting that they are each stimulated by the same biochemical pathway. Using cellular stratification approaches, we found that cortical granules are freely diffusible in the oocyte cytoplasm until meiosis. We also find that microfilaments provide the major pathway for cortical granule translocation to the cell cortex. In the future, these results will enable us to identify specific biochemical mechanisms that reactivate meiotic progression in the sea urchin oocyte, as well as the identification of the motive force for cortical granule translocation in this conserved process.

MATERIALS AND METHODS

Animals

Lytechinus variegatus were obtained from the Duke Marine Laboratory (Beaufort, NC), from Scott's Services (Miami, FL) or from Sue Decker (Miami, FL). Oocytes and eggs were handled as described (Berg and Wessel, 1997).

Reagents

The following chemical stocks were used: dibutyrl cAMP (DbcAMP) at 10 mg/ml in filtered artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH), isobutylmethylxanthine (IBMX) at 50 mM (1.1 mg/ml in ASW), theophylline at 10 mg/ml in ASW, Okadeic acid at 10 μg/ml in DMSO, and 6-dimethylaminopurine (6-DMAP) and lithium chloride, each at 1.0 M in filtered ASW (all from Sigma Chemicals, St Louis, MO).

Cytoskeletal inhibitors were used at the following concentrations: nocodozole, 10 μg/ml; colchicine, 10 μM; cytochalasin B, 1 μg/ml; and cytochalasin D 1 μg/ml; 2 μM latrunculin A (Calbiochem) (Schatten et al., 1986) (all in ASW with 100 μg/ml ampicillin). In cases where DMSO was used for the solution vehicle, DMSO was tested alone at the same dilution and was found to have no effect in the assays used here.

The following reagents were each from LC Labs (Woburn, MA): roscovitine (cat R-1234; inhibitor of $p34^{cdc2}$ in DMSO); olomoucine (cat O-3590; inhibitor of $p34^{cdc2}$ in DMSO); forskolin (F-99229; activator of adenylate cyclase that leads to increased intracellular cAMP, in DMSO); Rp-cAMPS (A-9684; blocks cAMP-mediated signal transduction by inhibiting PKA, and resistant to hydrolysis by phophodiesterases, stock in water); H-8 (H-3660; blocks PKA (and some PKG) activity, in water); and H-89 (H-5239; an inhibitor of PKA, in DMSO).

Oocyte handling and labeling

For tracking maturation of single oocytes, a 96-well flexible assay plate (Becton Dickinson, Oxnard, CA) was used. For culturing populations of oocytes, either 7 mm depression glass multi-well plates (previously treated with GelSlick; AT Biochem, Malvern, PA) or polypropylene dishes (fashioned from beaker bottoms) were used and incubations were performed in humid chambers. Oocyte cultures routinely consisted of filtered ASW with ampicillin (100 μg/ml), which was replaced every 12 hours. For visualization of cortical granules, oocytes or eggs were fixed in 3.7% formaldehyde in ASW for 20-30 minutes. They were then extracted by ice cold 100% methanol for at least 10 minutes and washed in ASW containing 0.1% Tween-20 (ASW-Tw20). Storage prior to labeling was always at 4°C in ASW with ampicillin (100 μg/ml). Cortical granules were then immunolabeled and quantified as described elsewhere (Berg and Wessel, 1997). We note that when washing the oocytes/eggs, especially in the microtiter plate incubations of single cell cultures, it is better to replace the solution, than to move the oocyte.

For visualization of microfilaments, oocytes and eggs were fixed in freshly prepared 4% paraformaldehyde in Ca^{2+}-free seawater (McClay, 1986) for 15 minutes, and then washed in ASW-Tw20. To label microfilaments, 3-5 μl Texas Red phalloidin (Molecular Probes, Eugene, Oregon) was added to 100 μl ASW with 0.5% Tween-20 (ASW-Tw20) for at least 15 minutes and the cells were then visualized.

Oocyte stratification

Isolated oocytes were resuspended in 28% sucrose to give a final concentration of 16% sucrose and placed in a flat-bottom microfuge tubes. The cells were then spun in an Eppendorf microcentrifuge either at low speed (1500 ***g*** for 10 minutes), to partially displace the cortical granules, or high speed (5000 ***g*** for 10 minutes), to completely stratify the granules in the centrifugal pole. To measure cortical granule displacement, cells were prepared for immunolabeling (fixed in formaldehyde) immediately after centrifugation. Quantitation of cortical granules in stratified or recovered cells was also performed as above, using measurements in the centrifugal and centripetal hemispheres. Displacement (%) is then calculated as the difference of the two values (each oocyte value is an average of three readings) over the total value of the area measurements. Areas of the germinal vesicle were excluded from measurement. For experiments testing cytoskeletal function, cells were pre-incubated with cytoskeletal inhibitors (see above) for 10 minutes before centrifugation and/or immediately after centrifugation for a given period in ASW/ampicillin.

To measure cortical granule displacement during meiosis, oocyte cultures were examined hourly, and cells having undergone GVBD were removed and stratified. Because of the inability to synchronize oocyte GVBD, we were not able to stage the meiotic progression accurately, but we were able to examine 128 cells in these experiments.

Endocytosis assays

Isolated oocytes were incubated in FM1-43 (Molecular Probes, Eugene, OR) at 1 μM. To evaluate endocytosis, oocytes were then

visualized by confocal microscopy using a Zeiss LSM 410 and endocytic activity was assessed quantitatively as for cortical granule translocation (see above).

In situ RNA hybridization

Oocytes were hybridized for transcripts of cleavage stage histones as described previously (Berg and Wessel, 1997). Briefly, oocytes and eggs were fixed in 2% glutaraldehyde and prepared for whole mount in situ hybridization (Ransick et al., 1993). Digoxigenin-labeled antisense transcripts were synthesized by first linearizing the tandemly-repeated histone clone (Holt and Childs, 1984) that was subcloned into pBluescript (Stratagene, LaJolla CA) with *Not*I, and then transcribed using T7 RNA polymerase. Controls for these experiments included use of sense strands and non-relevant transcript probes.

RESULTS

Regulation of meiotic maturation in cultured oocytes

Germinal vesicle breakdown (GVBD) and meiotic maturation occur spontaneously in sea urchin oocytes released in spawning or cultured after removal from the ovary. We find that only full-size oocytes mature and that once maturation begins it takes approximately 9 hours to complete (in *Lytechinus variegates*, at 22°C) (Berg and Wessel, 1997). As opposed to its close relative, the starfish, we have no control over when sea urchin oocytes begin maturation or any means to activate them to begin germinal vesicle breakdown. This technical shortcoming restricts the experimental protocols possible to study cortical granule translocation. However, the ability to monitor and manipulate thousands of oocytes is feasible.

When oocytes mature in vitro, the resulting eggs sometimes have pronuclear abnormalities (Fig. 1). One aberration seen is that the resultant egg contains two pronuclei. These twin pronuclei are equal in size to the normal pronucleus of an egg, and are always adjacent to each other. This has been observed in normal shed eggs (at frequencies sometimes approaching 0.5%), but this phenotype is always greater in in vitro matured eggs, and can routinely reach frequencies as high as 18%. Although these eggs have not been followed developmentally, the nuclei appear normal based on their size, presence of a nuclear envelope and transcriptional activity, as judged by accumulation of transcripts encoding cleavage-stage histones (Fig. 1E). Transcription of these genes is silent in oocytes but is activated in the pronucleus after meiotic maturation (Venezky et al., 1981). Surprisingly, each of these twin pronuclei often contain increased signal for the histone transcript, perhaps indicating a diploid nucleus.

The second aberration we have seen during in vitro oocyte maturation is in the size of the pronuclei. Approximately 25% of the eggs resulting from oocytes matured in vitro contain enlarged pronuclei (20-22 μm instead of the normal 12 μm pronuclei, Fig. 1). Again, we do not know the cause for this aberration or the ploidy of the nuclei, but they do accumulate near-normal levels of transcripts for the cleavage-stage histones (data not shown). Furthermore, in each case of these oocyte aberrations, the cortical granules still translocate, and exocytose in response to either sperm or Ca^{2+} ionophore indistinguishably from their in vivo matured siblings. This may indicate that the cortical granule translocation signals have occurred well before the aberration resulting in malformed pronuclei, or that meiotic maturation and cortical granule translocation are completely independent of each other.

To understand the mechanism that initiates and carries out cortical granule translocation, we attempted to manipulate meiotic maturation using a variety of reagents and assess the effect, if any, on cortical granule translocation. We tested several different approaches to either inhibit or to stimulate the process. While this repertoire is not exhaustive, it does include diverse reagent types potentially impacting multiple different pathways. We tested 1-methyladenine and similar compounds for their ability to activate maturation as they do in the closely related starfish (Kishimoto, 1999), but none of these reagents had any effect on stimulating maturation (or in delaying the process; data not shown).

cAMP levels in mammalian and frog oocytes are key to meiotic progression and artificially high cAMP levels high block germinal vesicle breakdown in these cells. To test the effect of cAMP in meiotic maturation in sea urchin oocytes, isolated oocytes were cultured in ASW/ampicillin with several reagents that keep the cAMP levels artificially high in the oocyte. These include dibutyrl cAMP, theophylline, IBMX, 5-DMAP and combinations thereof. The results show that each reagent that elevates cAMP levels had a dramatic effect in blocking maturation (Fig. 2A). For dbcAMP and theophylline,

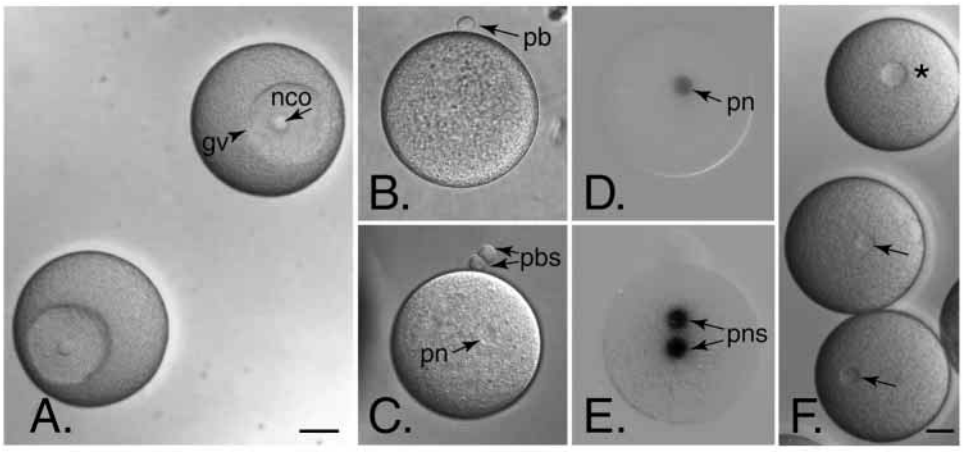

Fig. 1. Oocyte maturation in vitro in sea urchins begins with movement of the germinal vesicle to an asymmetric (future animal pole) position (A), followed by germinal vesicle breakdown and release of first one polar body (B), then a second polar body (C). Frequent aberrations in maturation in vitro include generation of two pronuclei (D, egg matured in vivo; E, egg matured in vitro – note two pronuclei, each transcriptionally active for cleavage stage histone genes), and formation of enlarged pronucleus (F, compare enlarged pronucleus * to normal-sized pronuclei, arrows; all matured in vitro). Scale bars: in A, 25 μm for A-E; in F to 25 μm in F. gv, germinal vesicle; nco, nucleolus; pb(s), polar body(s); pn(s) pronucleus.

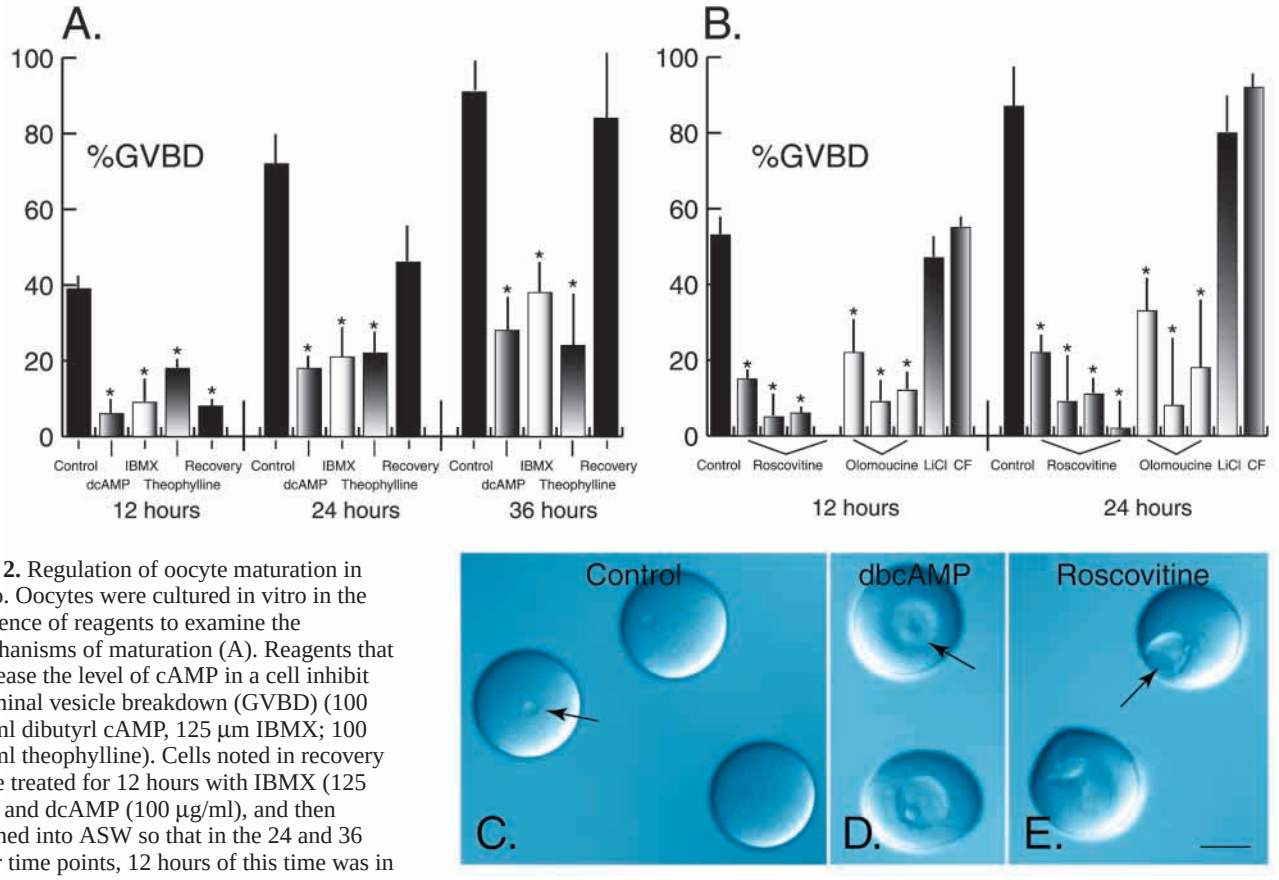

Fig. 2. Regulation of oocyte maturation in vitro. Oocytes were cultured in vitro in the presence of reagents to examine the mechanisms of maturation (A). Reagents that increase the level of cAMP in a cell inhibit germinal vesicle breakdown (GVBD) (100 μg/ml dibutyrl cAMP, 125 μm IBMX; 100 μg/ml theophylline). Cells noted in recovery were treated for 12 hours with IBMX (125 μm) and dcAMP (100 μg/ml), and then washed into ASW so that in the 24 and 36 hour time points, 12 hours of this time was in the inhibitor. (B) Roscovitine at 0.1, 0.5, 2 μM; olomoucine at 10, 30 and 90 μM; LiCl, at 30 μM; and CF, coelomic fluid at 50%. Asterisk indicates significantly different from control values at $P>0.01$ confidence level determined by Student's *t*-test analysis. (C) Control oocytes matured into eggs containing pronuclei (arrow), within 24 hours of incubation. (D) Sibling oocytes cultured for 24 hours in the presence of 100 μg/ml dibutyrl cAMP; note the distinct and centrally located germinal vesicle, arrow. (E) Sibling oocytes cultured for 24 hours in the presence of 0.1 μM roscovitine, note distinct but misshapened germinal vesicle, arrow. Scale bar: 50 μM.

dramatically increased concentrations (in excess of 400 μg/ml) showed neither adverse effect on the cells nor any additional inhibitory effect on GVBD. However, IBMX had a narrower range of useful concentrations: at 0.5 μM and above, cells became blebby and died within 12 hours of treatment. With these treatments, oocytes could be blocked from GVBD (greater than 50%) for 2 days or longer with no adverse effect. Key to this success though was a constant (every 12 hours) replacement of fresh media with the test reagent. Effective recovery of the oocytes from these reagents showed that the treatments did not compromise viability. Instead, the recovered oocytes actually showed enhanced synchrony of maturation. Perhaps some factor important for maturation accumulated during the culture period, and removal of the inhibitor was able to stimulate maturation in the population of competent oocytes. Although the mechanism is not known, this phenomenon may be useful technically in synchronizing or predicting maturation within a population.

Lithium was tested for its effect in maturation, as it was recently shown that GSK3b is involved in the progesterone-activation pathway in *Xenopus* oocytes (Fisher et al., 1999; Ferkey and Kimelman, 2000). At concentrations known to affect GSK3b activity in sea urchins and other embryos, however, we found no difference in the frequency of maturation detected in sea urchin oocytes relative to their sibling controls. Treatment of oocytes from 2-64 μM resulted consistently in 78-85% maturation in 14 hours, indistinguishable from controls. No difference from controls was seen in earlier time points either, also indicating no affect on the rate of maturation. These treatments were, however, effective at inducing exogastrulation in embryos, as predicted (data not shown) (Harvey, 1956).

We also tested the effect of coelomic fluid on oocyte maturation, reasoning that as the ovaries are directly bathed in coelomic fluid, that perhaps removing the ovary and culturing the oocytes in vitro in ASW may release the oocyte from an inhibition of maturation. Coelomic fluid was isolated from gravid females and the oocytes were bathed directly in 100%, 50% or 10% coelomic fluid. Oocytes were then monitored regularly. No differences in maturation rate, or overall percentages of maturation were observed, however, when compared with controls (Fig. 2B and data not shown). Thus, the maturation of oocytes we observed in vitro is not simply because of the release of an inhibitor present within coelomic fluid. Instead, it is possible that maturation is blocked by direct interaction between somatic cells of the ovary and oocytes by a cAMP-dependent pathway.

The cyclin-dependent kinase (Cdk) activity of maturation-

Table 1. Regulation of in vitro oocyte maturation: additional reagents tested

Reagent	Concentration	% of control
Forskolin	1 μM	46±16
	10 μM	16±10
	30 μM	14±6
6-DMAP	0.5 mM	3±1
	1 mM	4±1
RpcAMP	0.3 μM	91±17
	1 μM	67±8
	3 μM	72±13
H-8	2 μM	92±15
	10 μM	74±12
	50 μM	68±22
H-89*	1 μM	78±14

GVBD was assessed by microscopic observation and expressed as a percentage of the control value of that experiment at a single time point: after 24 hours culture in isolation. At least 200 cells were used in each experimental and control condition and each experiment was performed at least twice.

*Higher concentrations were lethal over the timeframe of the incubation, although for all experiments, wells were not included when greater than 10% of the cells died within that well.

promoting factor (MPF) is required for meiotic resumption in all oocytes examined, and is associated with cAMP-dependent activities (Grieco et al., 1996). Effective and selective inhibitors of Cdk1 include roscovitine and olomoucine (Meijer et al., 1997). Treatment of oocyte cultures with roscovitine resulted in striking inhibition in meiotic maturation (Fig. 2B,E and up to 48 hours, data not shown). While the germinal vesicle of roscovitine-treated oocytes remains intact with a distinct nucleolus, the germinal vesicle shape and position becomes asymmetrical (Fig. 2E). This phenotype is also observed when oocytes are treated with olomoucine (Fig. 2B and data not shown). While olomoucine inhibits Cdk1, it is less specific than is roscovitine, and is less efficacious in our assays (Fig. 2B) (Meijer et al., 1997). Additional reagents used to test the regulation of oocyte maturation, and their efficacy, are shown in Table 1.

Cortical granule translocation at meiotic maturation

Given the ability to inhibit GVBD by several different means, we next wanted to test if the initiation of cortical granule translocation and meiotic progression share a common biochemical pathway. Were these events separate, we reasoned that at least some of the treatments that inhibit meiotic maturation should not affect initiation of cortical granule translocation. Conversely, if the two processes are linked, then our repertoire of biochemical inhibitors should not be able to resolve them. To test these hypotheses, we treated oocytes as above and monitored cortical granule distribution. In no case did we ever see any detectable cortical granule translocation in a cell that has not re-initiated meiotic maturation (Fig. 3). This is particularly apparent in comparing cells treated to alter cAMP levels or MPF activity. In these experiments, we saw a small percentage of the cells escape the inhibitory treatment in meiosis and mature. In these cells, cortical granules also translocate, although no cells blocked from GVBD exhibit any cortical granule translocation. Conversely, in treatments that do not effect meiotic resumption, e.g. lithium chloride, cortical granule translocation is also not affected. In addition, we found that cortical granule translocation was always an all or none

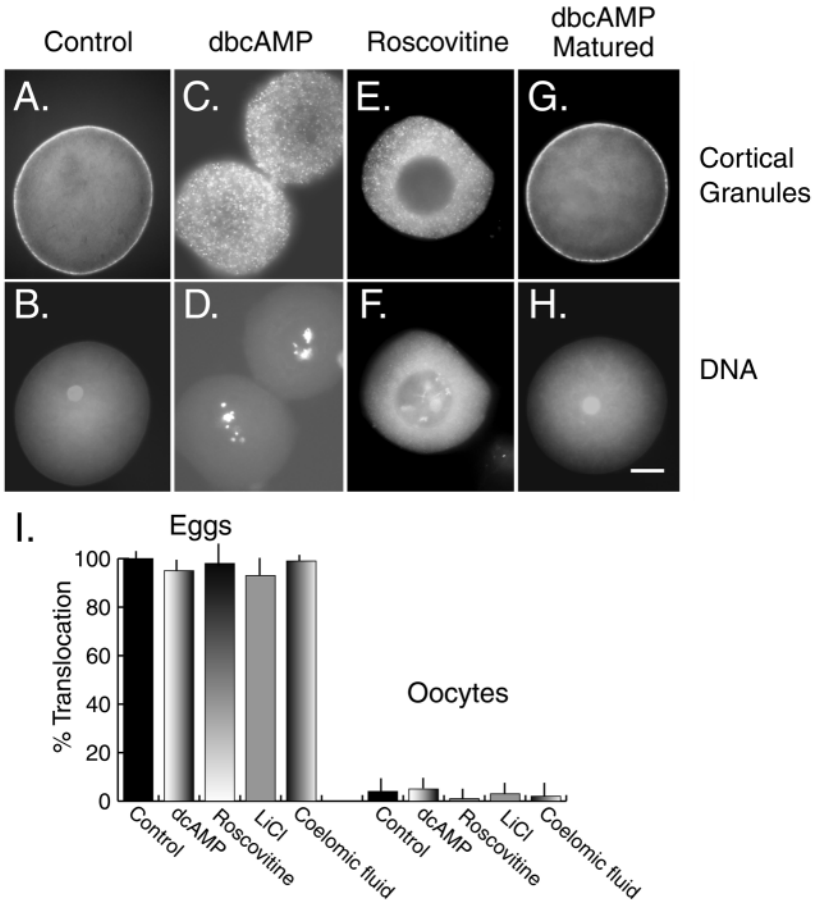

Fig. 3. Cortical granule translocation is linked to meiotic maturation. Immunolabeling cortical granules of a control cell (A) that has matured in vitro shows that nearly all of its cortical granules have translocated to the cortex. Its pronucleus, which is indicative of meiotic maturation, is evident as a uniform, 12 μm sphere, labeled with Hoechst (B). Cells inhibited from maturation by cAMP (C) or roscovitine (E) show prominent germinal vesicles with dispersed chromatin (D,F, respectively) and no translocation of cortical granules. A cell that did mature in the presence of dbcAMP (even though only a small percentage do, see Fig. 2) shows a normal looking cortical granule translocation (G) and a pronucleus (H). Scale bar: 25 μm. (I) Cortical granule translocation was quantitated by measuring immunolabeled cortical granules in eggs and oocytes cultured in vitro. Even though during some treatments a small percentage of the oocytes mature (see Fig. 2), those that do mature show translocation indistinguishable from control, whereas oocytes that have not matured by these same treatments also have no demonstrable translocation, indistinguishable from an oocyte freshly isolated from the ovary. None of the experimental treatments differ from the control at the $P>0.05$ confidence level by Student's *t*-test analysis.

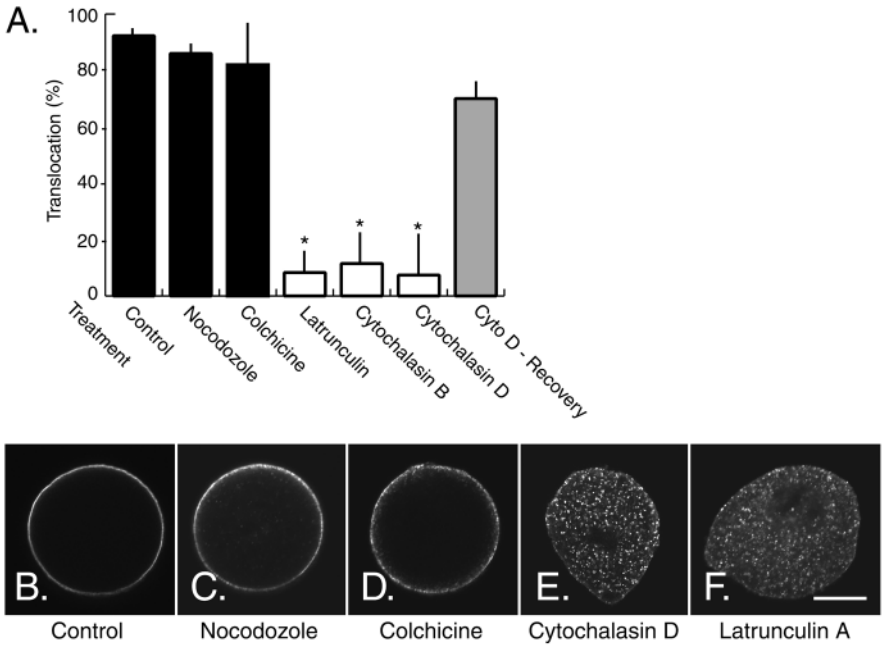

Fig. 4. Cortical granule translocation is microfilament dependent. Quantitation of cortical granule location following germinal vesicle breakdown shows that disruption of microfilaments severely reduces translocation. (A) Quantitation of cortical granule translocation (%) in the presence of various inhibitors. Error bars indicate ±1 s.d. and the asterisks indicate significantly different from control values at $P>0.01$ (confidence level determined by Student's *t*-test analysis). Treatment with either of two different microtubule inhibitors (nocodozole, C; colchicine, D) shows no significant difference in translocation from control cells (B), whereas treatment with any of three different microfilament inhibitors [cytochalasin D (E), cytochalasin B (data not shown), latrunculin A (F)] has a dramatic inhibitory effect. Recovery from treatment with cytochalasin D suggests that inhibition of translocation does not cause irreparable damage to the cell. Scale bar: 50 μm.

process. We never observed partial translocation in germinal vesicle-intact oocytes or mature eggs. As the oocyte treatments are diverse, with each yielding the same result, we believe that the cortical granule translocation mechanisms are either linked to MPF activity, or that one target of active MPF (or of the MPF activator) is in the cortical granule translocation machinery.

Cortical granule translocation is microfilament dependent

To examine the mechanism of cortical granule translocation, we examined oocytes cultured in the presence of a variety of cytoskeletal inhibitors. In these experiments, oocytes were isolated, cultured for 24 hours, and then processed for in situ immunolabeling of cortical granules. When the microtubule inhibitors nocodozole and colchicine were used, cortical granules translocated to the cortex generally as in controls (Fig. 4). When examined more carefully though, we found that the cortical granules did not form a perfect and tight monolayer at the plasma membrane. Instead, they were patchy, not tightly apposed to the plasma membrane, and not secretion competent (Fig. 4C,D). However, this translocation phenotype is in stark contrast to that observed in oocytes treated with microfilament inhibitors. While germinal vesicle breakdown proceeds as in controls, cortical granules do not translocate to the cell cortex and instead remain uniformly distributed throughout the cytoplasm.

Unfortunately, we are limited in our ability to directly visualize microfilament bundles in the cytoplasm internal to the actin band at the oocyte cortex, which is likely to be a problem related to the cell volume (Boyle and Ernst, 1989). Thus, we are also unable to visualize directly the orientation or formation of microfilaments, the effectiveness of the inhibitors, or the co-labeling of cortical granules with microfilaments. Surprisingly, though, we find that coincident with cortical granule translocation and, just before germinal vesicle movement at meiotic maturation, the germinal vesicle acquires a transient microfilament population inferred by phalloidin staining (Fig. 5; and data not shown for antibody labeling). Morphologically at this time, the germinal vesicle appears to become more 'stiff' or 'firmer' than normal. Although we do not know the functional relevance of this phenomenon, it is an extreme density of microfilaments in order for us to visualize it, and is an important indicator of

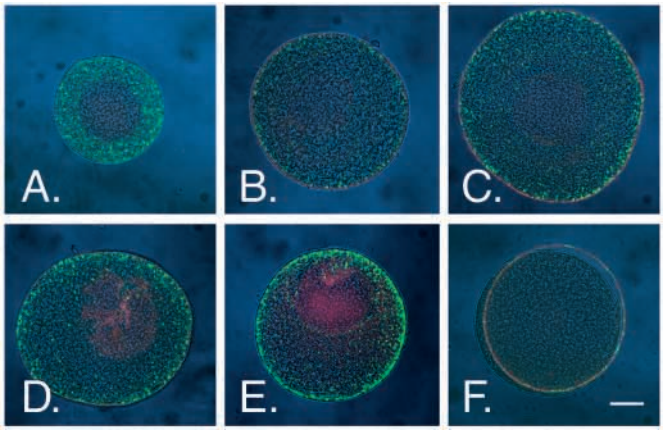

Fig. 5. Actin filament formation during cortical granule translocation. Actin filaments were visualized and inferred by phalloidin staining followed by immunolabeling for cortical granules. Shown are early (A), mid- (B) and full- (C) sized oocytes, which have predominantly cortical actin labeling (oocyte in C is slightly compressed to better reveal the germinal vesicle). As oocytes begin meiotic maturation (D,E), the cortical granules move toward the cortex, the germinal vesicle becomes decidedly asymmetrical and phalloidin positive. The germinal vesicle then breaks down and matures to a haploid nucleus, while the cortical granules complete formation of the monolayer at the cortex. Although we can not detect individual microfilament bundles, cortical granule translocation coincides with microfilament formation in the germinal vesicle. Scale bar: 25 μm.

cortical granule translocation. Perhaps microfilament formation and cortical granule translocation are early indicators of MPF activation just prior to GVBD.

We have assumed that microfilaments are present and functional for organelle motility within the cytoplasm of the oocyte to rationalize the observation that cortical granule translocation is blocked by microfilament inhibitors. However, we wanted to be able to distinguish between two possible explanations for the lack of cortical granule translocation in pre-meiotic oocytes. One explanation is that microfilaments that function in organelle transport do not form until meiotic maturation. In this case, the germinal vesicle microfilaments would be indicative of polymerization of cytoplasmic actin. An alternative explanation is that microfilaments are present in the cytoplasm of developing oocytes, and are functional in other processes, but either the cortical granules do not interact with them, or the granules have no active motor associated with them. To distinguish between these possibilities, we used endocytosis as a functional test of microfilament motive forces within the oocyte. Endocytosis is active in oocytes, at least in vivo, in order to accumulate the heterosynthetic macromolecules like the major yolk protein (MYP) (Scott and Lennarz, 1989). We also reasoned that internalization of endocytic vesicles in these oocytes is likely microfilament mediated, as has been shown recently in several cells, including yeast and mammalian cell lines (Jeng and Welch, 2001). Therefore, we functionally tested microfilament association with oocyte endocytosis activity and internalization of vesicles in vitro.

To assay endocytosis, we used two types of markers, each of which have previously been used in sea urchin eggs and embryos. These include FM1-43 (fluorescent lipophilic, membrane impermeant dye, as a marker for the fate of internalized plasma membrane), and rhodaminated dextran as a fluid-phase marker (Whalley et al., 1995). We isolated oocytes and cultured them with FM1-43 and/or rhodaminated dextran in the presence or absence of cytoskeletal inhibitors, and then imaged the live oocytes by confocal microscopy. When endocytosis was compared between the different cell treatments, we found first that oocytes are very active in endocytosis throughout oogenesis in vitro, compared with eggs and embryos (Fig. 6 and data not shown). Second, oocyte endocytosis is insensitive to cAMP levels (data not shown); in either the presence or absence of cAMP levels that inhibit meiotic maturation, endocytosis is equivalent. This result negates the possibility that treatment regimes on meiosis block cortical granule translocation by an indirect affect on microfilament function. Third, internalization of endocytic vesicles is insensitive to microtubule disruption, but completely dependent on the presence of microfilaments (Fig. 6). Cells were treated with cytochalasin B, latrunculin A, jasplackinolide or cytochalasin D (Fig. 6 and data not shown) to disrupt microfilament function, or with colchicines to disrupt microtubule function. Note that surface labeling of the oocyte by FM1-43 appears unaffected in any treatment relative to control cells. Internalization of label is, however, dramatically reduced or eliminated when cells are treated to disrupt microfilaments, whereas colchicine-treated oocytes endocytose normally (Fig. 6). These results show that microfilaments and microfilament-mediated motors are functionally active in oocytes to move specific vesicle types. We conclude that cortical granules do not translocate because they either lack a linkage to the microfilaments or they lack a functional motor with which to travel on the microfilament. The lack of translocation is not because of a global absence of microfilaments or of microfilament-motor function in these oocytes.

Cortical granules associate with microfilaments at meiosis

The above results suggest that during meiosis, cortical granules associate with microfilaments for translocation to the cell surface. If this model is correct, then we predict that during oogenesis, cortical granules are either not linked to microfilaments and freely mobile within the cytoplasm or are linked to microfilaments but have no motor function. To test this model, oocytes at different stages of development were centrifuged in an isopycnic sucrose gradient to stratify their organelles under either relatively low or high centrifugal forces (1500 ***g*** and 5000 ***g***, respectively, for 10 minutes). During centrifugation, the organelles of the cell stratified according to their relative density, such that cortical granules were most centrifugal, followed centripetally by mitochondria, yolk platelets, endoplasmic reticulum and Golgi membranes, lipid droplets, and then the germinal vesicle. In stratified oocytes, we documented the positioning of cortical granules and yolk platelets (by immunolabeling), mitochondria and nuclei (by Hoechst labeling), but only inferred the membrane/lipid region by appearance in the microscope (data not shown). This is consistent with the stratification of organelles in eggs described elsewhere (Harvey, 1956), except that in eggs the cortical granules are attached to the plasma membrane and are not displaced. When cortical granules are first undocked from the egg plasma membrane by chemical treatment and then

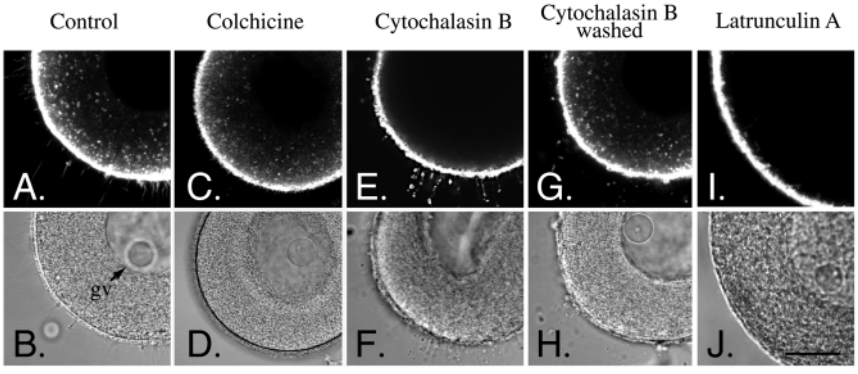

Fig. 6. Microfilaments are functional in oocytes prior to GVBD and cortical granule translocation. Shown are oocytes treated for 30 minutes with the given reagent and then 10 minutes with FM1-43 (top) and DIC (bottom). Images were recorded by confocal microscopy. The plasma membrane is intensely labeled in all cells because of exposed surface area. Endocytosis is inhibited reversibly in latrunculin A and cytochalasin B, respectively, but no inhibition is seen in cells treated to disrupt microtubules (C). Scale bar: 25 μm. The endocytic vesicles are approximately 0.5 μm in diameter.

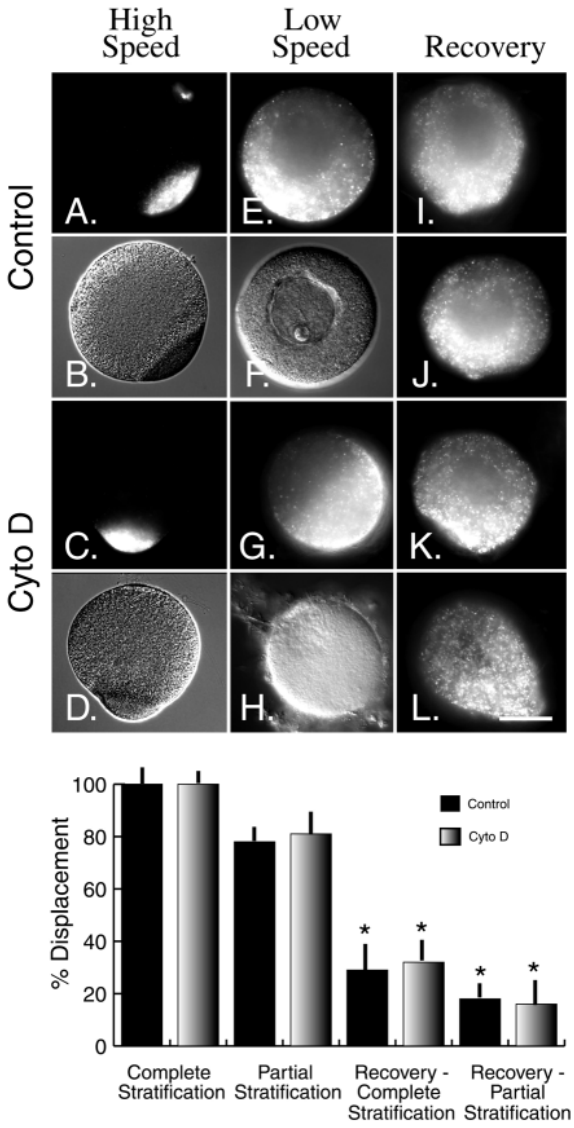

Fig. 7. Cortical granules are not associated with structural microfilaments prior to translocation. Oocytes were subjected to isopycnic sucrose centrifugation to stratify organelles. (A) Immunolabeled cortical granules of a control cell (B) after high-speed stratification (5000 ***g***). Note refractile band of cortical granules in B, corresponding to immunolabeled cortical granules in A. Same scheme for C,D, except that prior to stratification, cells were treated with cytochalasin D, resulting in a slightly bulged centrifugal end, sometimes exaggerated for many μm (data not shown). We interpret this disfiguration to reflect the loss of cortical microfilaments. (E) Immunolabel and (F) DIC image of low-speed spin (1500 ***g***) in control (E,F), or (G,H) cytochalasin conditions. Conditions were sought that would maximize resolution of the cortical granule displacement, but no difference was seen. (I-L) Cells allowed to recover in control conditions after low-speed spin (I,K, recovered for 30 minutes) or from a high-speed spin (J,L, recovered for 2 hours). (Below) Graphical representation of cells represented above, error bars represent ±1 s.d.; scale bar: 50 μm. At least 10 cells were assessed for each condition. None of the experimental conditions produce result that are significantly different from control values at $P>0.10$ (confidence level determined by Student's *t*-test analysis), but each of the recovery conditions from stratification is significantly different from the stratification alone (*), even in the presence of cytochalasin D.

stratified, the organelle profile is very similar to that seen in these oocytes (Hylander and Summers, 1981).

When oocytes were first treated with cytochalasin D (Fig. 7C,D,G,H, or data not shown for latrunculin A), we found that under high centrifugal conditions, nearly 100% of the cortical granules were displaced to the centrifugal most pole of the cell, indistinguishable from control cells. Following microfilament disruption, the cells became slightly distorted (note the bulge in Fig. 7D and compare with 7B) reflective of a loss in cortical microfilament integrity. Stratification under lower speeds (1500 ***g*** for 10 minutes) resulted in only partial displacement of the cortical granules in control cells (Fig. 7E,F), enabling a more-sensitive assessment of the granule mobility in the cytoplasm. Disruption of the microfilaments, however, caused no additional mobility of either cortical granules (Fig. 7G,H) or the other organelles examined (data not shown). Thus, cortical granules in oocytes do not appear to be linked to microfilaments or microfilament-associated structures in a way that would retard their mobility in the cytoplasm.

Additional evidence to suggest that the cortical granules are freely diffusible in the cytoplasm comes from recovery experiments. Oocytes stratified under either high or low centrifugation conditions were cultured in vitro for different times and the position of their cortical granules was assessed. Surprisingly, we found that in control oocytes, the cortical granules returned to a uniform distribution relatively quickly. Fig. 7 shows a control oocyte 30 minutes after low-speed centrifugation (Fig. 7I) or 2 hours after a high-speed spin (Fig. 7J). In fact, after prolonged incubation and subsequent oocyte maturation, cortical granules translocate normally to form a monolayer in the egg cortex and normal cortical granule exocytosis follows fertilization or ionophore treatment (data not shown). Thus, the stratification protocol does not compromise the function of the cortical granules, but instead appears to reflect their normal condition in the cytoplasm. Were this re-dispersion of cortical granules throughout the cytoplasm instead mediated by a transient cytoskeletal interaction, we should be able to disrupt this recovery by cytoskeletal inhibitors and have a new assay system to study cortical granule movement. Stratified oocytes were thus cultured with inhibitors of both microfilaments and microtubules, but, as expected, neither had any impact on the re-dispersion of cortical granules in the oocytes (Fig. 7K,L, and data not shown). This was the case regardless of whether the oocytes were treated only after the centrifugation step, or whether they were also treated before stratification. The only noticeable difference in the cytochalasin-treated oocytes from controls is that slightly distorted oocytes did not usually recover a uniform spherical shape (see Fig. 7L, for example). This probably reflects the loss of integrity of the cortical microfilaments and the role they play in the shape of these cells. From these stratification and recovery experiments, we conclude that cortical granules are freely diffusible in the oocyte cytoplasm.

The extension of the above hypothesis is that cortical granules do associate with microfilaments at meiosis when they translocate to the cortex. This linkage would presumably impede their displacement. To test this hypothesis, oocytes that had undergone GVBD (after GVBD but before formation of the pronucleus) were stratified, and compared with both stratified GVBD-sibling oocytes treated with cytochalasin D and to sibling oocytes prior to GVBD (Fig. 8). Under low-

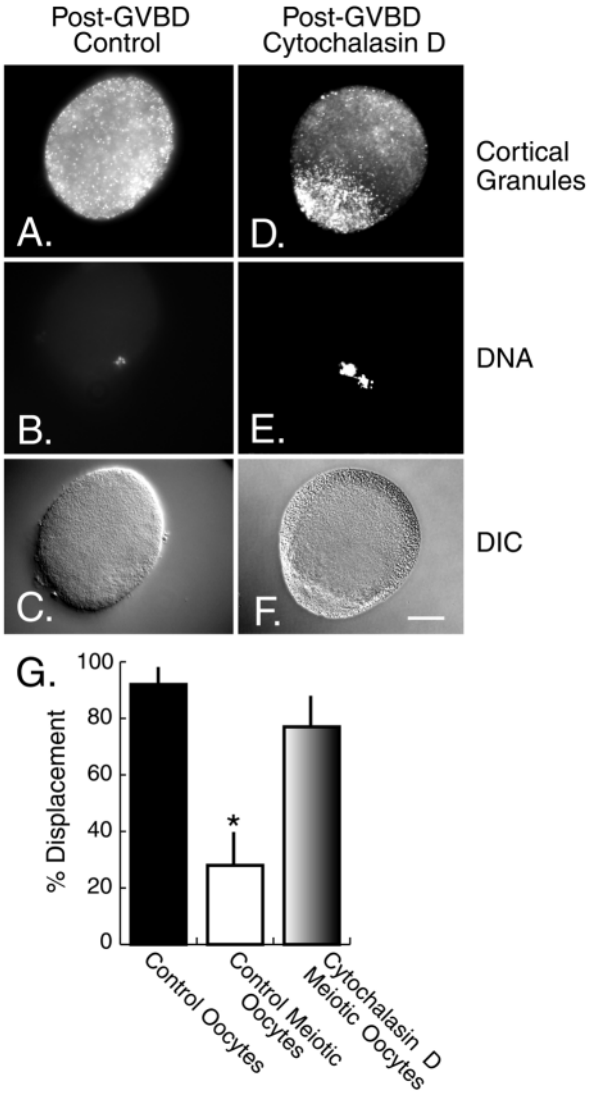

Fig. 8. Cortical granules associate with microfilaments at GVBD. Oocytes having undergone GVBD in vitro were subjected to stratification either in the absence (A-C) or presence (D-F) of cytochalasin D. Cortical granules were then immunolabeled and their position quantified. (A,D) immunolabeled cortical granules; (B,E) Hoechst label; (C,F) DIC images. Control oocytes are as in previous figures, prior to GVBD. Meiotic oocytes have undergone GVBD, although the precise stage of meiosis is not known. (G) Quantitation of multiple experiments; asterisks indicate changes that are significantly different from control oocytes at $P>0.01$ (confidence level determined by Student's *t*-test analysis). Scale bar: 25 μm.

speed centrifugation, oocytes stratified during meiosis show very little displacement of their cortical granules (26.5%; Fig. 8A,G). However, meiotic oocytes treated with cytochalasin D for 10 minutes prior to centrifugation show markedly elevated levels of displacement (76.5%; Fig. 8D,G), significantly different from control meiotic oocytes, and more closely aligned with pre-meiotic oocytes. This change in mobility of cortical granules associated with microfilaments suggests that cortical granules first become associated with microfilaments at meiotic maturation and these microfilaments then direct their translocation to the cell surface.

DISCUSSION

The timing of cortical granule translocation from the cytoplasm to the cell surface is crucial to an egg. This is especially important for the sea urchin, where 15,000 cortical granules, each 1 μm in diameter form a dense monolayer at the cortex of the mature egg. If this were to occur before meiosis, it might restrict the internalization of heterosynthetic products such as the major yolk protein and compromise the viability of the zygote. Conversely, were the granules not to reach the plasma membrane prior to fertilization, a delay in exocytosis would probably lead to an increased incidence of polyspermy. Evidence presented in this work provides insight into the mechanisms that regulate the timing of cortical granule transport. We find that: (1) cortical granule translocation is coupled to meiosis, a parsimonious strategy to enhance coordination; (2) cortical granules are freely diffusible in the pre-meiotic cytoplasm, but associate with microfilaments early in meiotic resumption to translocate to the cell surface; and (3) formation of the cortical granule monolayer at the cell surface is simply not a result of trapping freely diffusible cortical granules. While this latter hypothesis was attractive considering the identification of complimentary SNARE proteins on the cortical granules of these and other oocytes (Wessel et al., 2001), it appears that cortical granules are actively transported to the cell surface and that they then attach by a yet unknown process. We believe that attachment to the cell surface must also be regulated and not possible until GVBD.

Currently, we do not know the trigger for initiation of cortical granule translocation but we will need to consider three likely candidates that may be mutually inclusive. These are: (1) cortical granule motor activation, (2) cortical granule attachment to a motor protein and/or (3) assembly of a microfilament pathway for cortical granule transit. Although our assay for microfilament association is at a macro-level at this point, it is currently not possible to better resolve any association between cortical granules and microfilaments in situ; even were we able to image individual microfilament bundles, a co-labeling of microfilaments with cortical granules does not reveal a functional association.

The mechanism of vesicle cargo linkage to cytoskeletal motors is not well understood, but recent results are intriguing in light of our results here with cortical granules. Members of the Ras superfamily of proteins, in particular Rab-GTPases, are present on organelles and function as part of a recognition complex for motor proteins (reviewed by Deacon and Gelfand, 2001). Evidence derived from both genetic and biochemical approaches point to a Rab-myosin V interaction in directing vesicle movement. This is wonderfully shown in a Rab27a-myosin V interaction in the transport of melanosomes from melanocytes to keratinocytes (Deacon and Gelfand, 2001; Scott and Zhao, 2001; Wu et al., 2001). Inhibition of this interaction results in a decrease or loss of keratinocyte pigmentation. In addition, the gene responsible for the human disease Griscelli syndrome was recently shown to be *RAB27A*. Individuals with a mutation in this gene experience partial albinism, as a result of defects in pigment transport mechanisms. In the context of cortical granule function, this is also important. Although cortical granules translocate perfectly well without microtubules, the cortical granules do not

distribute and dock properly at the cell cortex and therefore do not exocytose upon normal stimulation. One conclusion of this phenotype is that a myosin motor functions in getting the cortical granules to the cortex, and a microtubule motor serves as a molecular parking attendant, to distribute and dock cortical granules uniformly.

What is the putative microfilament motor involved in cortical granule movement? Currently we do not know, but we will focus on a member of the non-conventional myosin family, myosin V. The C-terminal tail of this motor protein appears to attach to vesicles for transport in a microfilament dependent process, via a Rab-GTP mediated process (Deacon and Gelfand, 2001). Furthermore, it was recently shown that melanosomes contain Rab3a and that this isoform is required for melanosome movement to the cell surface. Of interest here in sea urchin oocytes is that a Rab3 homolog is found abundantly and selectively on cortical granules throughout their biogenesis, translocation, docking and fusion (Conner and Wessel, 1998). Thus, one model to test in the future is the association of sea urchin myosin-V with the cortical granule Rab3. A functional association of myosin with vesicles appears to be regulated by a cell-cycle dependent kinase. For example, myosin V can be released from cargos by phosphorylation directly by CaMKII, and engage instead in mitosis- (or meiosis-?) specific activities (Cheney and Rodriguez, 2001). Specifically, CaMKII activity is implicated in cortical granule translocation in mice oocytes (Abbott and Ducibella, 2001; Abbott et al., 2001). Movement of the cortical granules to the cortex in mice is also suggested to occur via microfilaments: translocation is inhibited by cytochalasin D, but not by inhibitors of microtubule dynamics (Connors et al., 1998). Although these studies were also performed in static, fixed oocytes, it does suggest a conserved process between chordates and echinoderms.

The mechanism of initiating cortical granule translocation has also been examined in oocytes of starfish. In the closely related oocytes, 1-methyladenine stimulates activation of MPF to reinitiate meiosis. 1-Methyladenine has no effect on sea urchin oocytes, but in starfish it triggers Ca^{2+} release into the cytoplasm via Ins(1,4,5)P_3 receptors, resulting in major microfilament changes (Heil-Chapdelaine and Otto, 1996; Santella et al., 1999). Although cortical granule translocation was not quantitated in these studies, observed changes in the microfilaments include increased polymerization throughout the cytoplasm, as well as increased cortical and germinal vesicle microfilaments. It is noteworthy that these changes occurred prior to germinal vesicle breakdown, and corresponds to the timing of initiation of cortical granule translocation in the sea urchin oocyte.

The presence of microfilaments in the germinal vesicles of the sea urchin oocytes is intriguing for several reasons: (1) the transient nature of their appearance; (2) the coincidence of their presence with initiation of cortical granule translocation; (3) the fact that they are found at a time when the germinal vesicle is moving to the animal pole; and (4) that they are correlated with a change in the germinal vesicle shape and pending GVBD (Stricker and Schatten, 1991). As actin was localized in the present study by both phalloidin and antibody labeling in situ, and as both approaches have identified a transient population of actin filaments in the germinal vesicle, a step in actin nuclear localization, rather than polymerization, is likely to be limiting. A stage-dependent accumulation of actin in germinal vesicles has also been reported in frog oocytes (Parfenov et al., 1995), where it is believed that microfilaments contribute to nuclear vesiculation. In addition, several regulators of actin polymerization are also present in the nucleus or translocate to the nucleus (Rando et al., 2000), suggesting that there is some function of actin in the nucleus that is subject to regulation. For example, Zhao et al. (Zhao et al., 1998) find actin and actin-related proteins in transcriptional complexes of mammalian cells. This has also been seen in yeast, with genetic evidence supporting actin function both in regulating transcription and chromatin remodeling (Olave et al., 2002).

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