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Phosphorylated MARCKS: A novel centrosome component that also defines a peripheral subdomain of the cortical actin cap in mouse eggs

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Abstract

MARCKS (myristoylated alanine-rich C-kinase substrate) is a major substrate for protein kinase C (PKC), a kinase that has multiple functions during oocyte maturation and egg activation, for example, spindle function and cytoskeleton reorganization. We examined temporal and spatial changes in p-MARCKS localization during maturation of mouse oocytes and found that p-MARCKS is a novel centrosome component based its co-localization with pericentrin and γ-tubulin within microtubule organizing centers (MTOCs). Like pericentrin, p-MARCKS staining at the MI spindle poles was asymmetric. Based on this asymmetry, we found that one end of the spindle was preferentially extruded with the first polar body. At MII, however, the spindle poles had symmetrical p-MARCKS staining. p-MARCKS also was enriched in the periphery of the actin cap overlying the MI or MII spindle to form a ring-shaped subdomain. Because phosphorylation of MARCKS modulates its actin crosslinking function, this localization suggests p-MARCKS functions as part of the contractile apparatus during polar body emission. Our finding that an activator of conventional and novel PKC isoforms did not increase the amount of p-MARCKS suggested that an atypical isoform was responsible for MARCKS phosphorylation. Consistent with this idea, immunostaining revealed that the staining patterns of p-MARCKS and the active form of the atypical PKC ζ/λ isoform(s) were very similar. These results show that p-MARCKS is a novel centrosome component and also defines a previously unrecognized subdomain of the actin cap overlying the spindle.

Keywords: PKC; Mouse egg; MARCKS; Cortical actin; Meiosis; Centrosome; MTOC

Introduction

Both mouse oocyte maturation and egg activation are perturbed by manipulating protein kinase C (PKC) activity. For example, activators of PKC such as biologically active phorbol esters or diacylglycerols inhibit germinal vesicle breakdown (GVBD) (Bornslaeger et al., 1986) and induce cytoskeletal rearrangements (Capco et al., 1992; Gallicano et al., 1993; Moore et al., 1995). In oocytes derived from LTXBO mice, which aberrantly enter interphase after meiosis I, inhibiting PKC with bisindolylmaleimide I (BIM) promotes progression to MII (Viveiros et al., 2001). Treating metaphase II (MII)-arrested eggs with PKC activators results in cortical granule exocytosis (Endo et al., 1987), one of the earlier hallmarks of egg activation. Although these results indicate multiple functions for PKC, the targets for the action of PKC phosphorylation in the oocyte are not known.

The myristoylated alanine-rich C-kinase substrate protein, MARCKS, is one of the most predominant intracellular substrates for PKC (Aderem, 1992). MARCKS contains three highly conserved domains. The first domain at the amino terminus contains the consensus sequence for myristoylation and is responsible for association of MARCKS with lipid membranes. The second domain, also known as MH2 domain, resembles the cytoplasmic tail of the cation-independent mannose-6-phosphate receptor but its function is unknown. The third domain is referred to as the

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effector domain (ED) or phosphorylation site domain (PSD) and contains three serine residues that can be phosphorylated by PKC. When not phosphorylated this domain can bind calmodulin with high affinity, crosslink microfilaments in vitro (Hartwig et al., 1992; Swierczynski and Blackshear, 1995) or bind to PIP_2 (Sundaram et al., 2004). PSD phosphorylation results in loss of these activities, and frequently is accompanied by translocation from the membrane to the cytosol (Arbuzova et al., 2002). Modulation of PSD phosphorylation likely provides a mechanistic linkage between PKC activity and MARCKS function.

There are many isoforms of PKC in the oocyte, some of which have been localized to specific structures (Page Baluch et al., 2004). For example, PKC-δ associates with the meiotic spindles during maturation (Viveiros et al., 2003), whereas other PKC isoforms associated with the plasma membrane translocate to the cytoplasm following egg activation (Gallicano et al., 1995). The spatial restriction of PKC isoforms suggests that MARCKS phosphorylation, and presumably MARCKS function, is spatially regulated during maturation and following egg activation. We tested this idea by monitoring phosphorylated MARCKS (p-MARCKS) during oocyte maturation and following fertilization by confocal microscopy. We report that MARCKS is found throughout the oocyte and egg with no obvious change in its localization during maturation and following fertilization. In contrast, p-MARCKS displays distinct and unanticipated localizations that include the nucleus and a previously unrecognized subdomain of the actin cap overlying the MII spindle. Last, the co-localization of p-MARCKS with γ-tubulin and pericentrin indicates that it is a novel centrosome component.

Materials and methods

Oocyte and embryo collection and culture

Female CF-1 mice (6–8 weeks old) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Fully grown, cumulus cell-free, GV-intact oocytes were obtained from PMSG-primed female mice and collected in bicarbonate-free Minimal Essential Medium supplemented with pyruvate (100 μg/ml), gentamicin (10 μg/ml), polyvinylpyrrolidone (3 mg/ml), and 25 mM HEPES, pH 7.2 (MEM/PVP) as previously described (Manejwala et al., 1986). When needed to prevent GVBD, milrinone (final concentration 2.5 μM) was added. Oocytes that had undergone GVBD were collected 2.5–3 h after hCG injection. Metaphase I (MI) and metaphase II (MII) eggs were obtained 7 and 14 h after hCG injection, respectively. MI eggs were collected from the stimulated ovaries, whereas MII eggs were collected from the oviducts. In both cases, cumulus cells were dispersed with 0.3% hyaluronidase. One-cell embryos were collected 14–15 h or 20–22 h after hCG injection and mating to B6D2F1/J males (Jackson Laboratory, Bar Harbor, ME). To obtain oocytes that had undergone GVBD in vitro, fully-grown GV-intact oocytes were washed free of milrinone and cultured for 1 and 2 h in CZB medium (Chatot et al., 1989).

Immunocytochemistry

The following primary antibodies were used for immunocytochemistry at the indicated final concentrations: anti-p-MARCKS (10 μg/ml; Sigma, St. Louis, MO), anti-phospho PKC ζ/λ (6.2 μg/ml; Cell Signaling Technology, Beverly, MA), anti-pericentrin (2.5 μg/ml; BD Biosciences, San Jose, CA), anti-γ-tubulin (16 μg/ml; Sigma), anti-β-tubulin (7 μg/ml; Sigma). A rabbit antiserum that recognizes MARCKS and the corresponding preimmune serum were the generous gift of J.P. Blackshear (NIEHS); both were used at a 1:100 dilution for immunocytochemistry. The following secondary antibodies were used at the indicated concentrations: Alexa Fluor 488-conjugated goat anti-mouse IgG (4 μg/ml; Molecular Probes, Eugene, OR), and Cy 5 conjugated donkey anti-rabbit IgG (15 μg/ml; Jackson Immunoresearch, West Grove, PA). Specificity of immunostaining was demonstrated by using the antibody after incubation with an appropriate blocking peptide at 4°C overnight. The peptides were used at the following concentrations: pPKC ζ/λ blocking peptide, 40.3 μg/ml (Santa Cruz Biotechnology, Inc.); phospho-MARCKS peptide (phosphorylated at serine 152/156: KRFpSFKKpSFKLSG), and non-phospho-MARCKS peptide (KRFSFKKSFKLSG), 50 μg/ml each (Biosource, Hopkinton, MA).

The *zona pellucida* was removed by brief incubation in acidic Tyrodes solution, pH 2.5, prior to fixation. For p-MARCKS, cells were fixed in 2.2% paraformaldehyde in PBS for 40 min. When indicated, cells were fixed in the presence of 1 μM taxol to stabilize microtubules. After washing in blocking solution (BS; 0.1% BSA and 0.01% Tween-20 in PBS), cells were permeabilized for 15 min in BS containing 0.1% Triton X-100, washed and then incubated with the primary antibody diluted in BS for 1 h. The samples were then washed and incubated in the appropriate secondary antibodies for 1 h. The samples were then washed in BS and mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 1.5 μg/ml propidium iodide to visualize chromosomes. For actin staining, cells were incubated with phalloidin-Alexa Fluor 633 (Molecular Probes) for 15 min following the secondary antibody incubation.

Unless otherwise stated, each experiment was performed at least three times and each group analyzed contained a minimum of 20 cells. All cells were observed with a Leica TCS NT confocal microscope. Most images shown are sections through the cell region that included either chromatin or spindle, but in some cases, surface views are shown.

Immunoblotting

Protein extracts from equal numbers (50) of GV-intact oocytes, MII eggs, and 1-cell embryos were separated on a

10% SDS-PAGE gel, transferred to Immobilon-P, and immunoblotted according to standard procedures. The MARCKS antiserum was used at a final dilution of 1:1000, and the secondary antibody was an alkaline phosphatase conjugated anti-rabbit IgG (Jackson Immunoresearch). Immunoreactive proteins were detected using Vistra reagent (Amersham, CA) and the image captured using a phosphorimager.

Results

Alterations in localization of MARCKS/p-MARCKS during meiotic maturation

We first determined the localization of MARCKS from the GV-intact oocyte to 1-cell embryo stages (Fig. 1). In GV-intact oocytes MARCKS was preferentially located in the cytoplasm but was also found in the GV. We consistently observed by optical sectioning multiple (3–5) brightly staining spots in the GV (Fig. 1A) that could be speckles based on their size, location, and number. In MII eggs MARCKS staining was diffuse throughout the cytoplasm, including the region of the spindle, and similar staining was observed in 1-cell embryos with additional staining in the pronuclei (PN; Figs. 1C, E). There was no obvious difference in staining intensity at these stages, and this was confirmed by immunoblot analysis (Fig. 2).

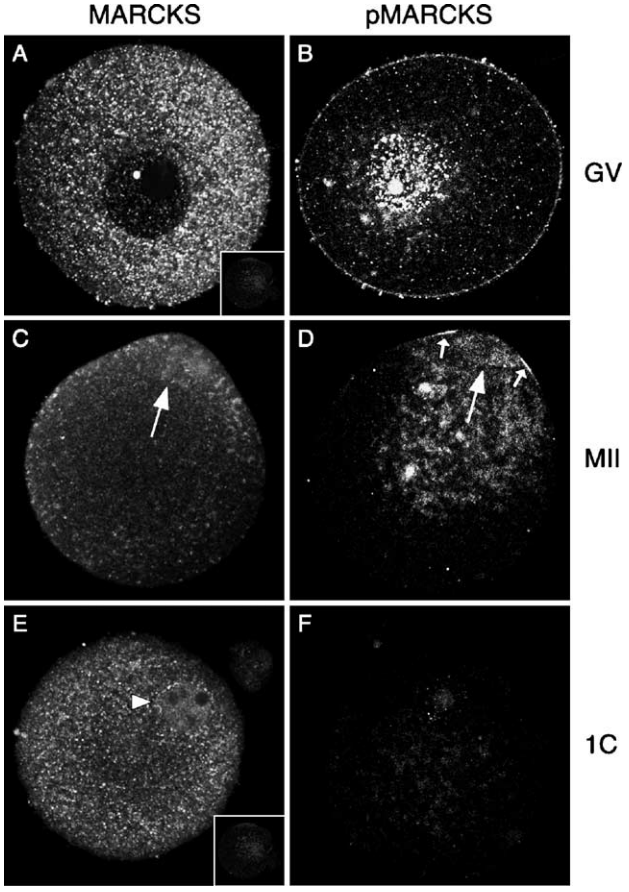

Fig. 1. Immunolocalization of MARCKS and p-MARCKS proteins. GV-intact oocytes (A, B), MII eggs (C, D) and 1-cell embryos (E, F) were immunostained using anti-MARCKS (A, C, E) or anti-p-MARCKS (B, D, F). The inset in panel A is an oocyte stained with the equivalent dilution of preimmune serum, and the inset in panel E is a 1-cell embryo stained only with secondary antibody. The large arrows indicate the region of the MII spindles, the small arrows indicate regions of cortical p-MARCKS staining, and the arrowhead indicates the female pronucleus; the male pronucleus had a similar appearance but is not in the confocal plane.

In contrast to MARCKS, p-MARCKS displayed distinct stage-dependent localizations, as well as overall decrease in signal intensity (Figs. 1B, D, F). In GV-intact oocytes, p-MARCKS was preferentially localized in the GV and oocyte cortex. It should be noted that this GV-staining pattern was observed in ~75% of the oocytes, whereas the remainder had no apparent nuclear staining. Moreover, these two GV-staining patterns were not correlated with the surrounded/nonsurrounded DNA configuration around the nucleolus (Bouniol-Baly et al., 1999). In MII eggs, p-MARCKS was enriched in the peripheral regions of the cortical actin cap that overlies the MII spindle. Little, if any, signal was observed in the 1-cell embryo. The p-MARCKS immunostaining was specific because an inhibitory phosphopeptide, but not the corresponding non-phosphorylated peptide, significantly reduced the signal (Fig. 3). Immunoblot analysis of p-MARCKS was not possible, because the antibody was not sensitive enough to detect the amounts of material that were readily obtainable.

These changes in p-MARCKS localization prompted us to monitor them more precisely during maturation (Fig. 4). To do this, the confocal settings were chosen to highlight localization at each stage, rather than being the same at all stages to monitor changes in amount as was done for Fig. 1. Following GVBD, p-MARCKS clustered around the condensing/condensed chromosomes and remained symmetrically located in the cortex. At MI, p-MARCKS appeared localized at the spindle poles and was preferentially in the cytoplasm surrounding the MI spindle. In addition, some of the cytoplasmic foci were reminiscent of microtubule organizing centers (MTOC) (Schatten et al., 1985). p-MARCKS was also enriched in the cortex overlying the MI spindle particularly at the spindle poles (Fig. 6A,B; this staining is not seen in the confocal section shown in Fig. 4). By MII, almost all of the p-MARCKS was localized in the region of the MII spindle and p-MARCKS staining at the spindle poles was intense. At this stage, cortical staining over the spindle poles was also observed. In the early 1-cell embryo, cortical localization was maintained in the region overlying the chromosomes but no staining was observed at the spindle poles (Fig. 4E). Furthermore, p-MARCKS was highly enriched in the actin-rich fertilization cone containing the sperm DNA as well as in the second polar body. Surface views of two MII-arrested eggs clearly reveal a peripheral ring-shaped subdomain of p-MARCKS

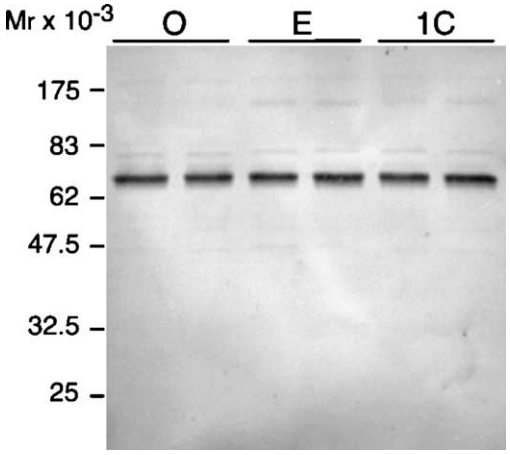

Fig. 2. Immunoblot of total MARCKS protein. Protein extracts of GV-intact oocytes (O), eggs (E), and 1-cell embryos (1C) were immunoblotted using anti-MARCKS. No apparent difference in total MARCKS protein was observed following oocyte maturation or fertilization. The experiment was performed 3 times and a representative blot is shown.

within the cortical actin cap (Figs. 4G and H). As described in the accompanying paper, this subdomain is the reciprocal of PAR-3, which occupies the central region of the cortical actin cap.

p-MARCKS is a novel centrosome component

The presence of p-MARCKS at the ends of the spindle and in cytoplasmic foci suggested that p-MARCKS is a centrosome component. Centrosomes, which are the principal MTOC's (Compton, 2000), consist of a pair of centrioles surrounded by a centrosomal protein matrix. In the mouse, centrioles are absent in the oocyte (Szollosi et al., 1972) and their function is replaced by MTOC's, which are immunologically related to the centrosomal matrix surrounding centrioles (Merdes and Cleveland, 1997) and support meiosis and early cleavage divisions. Pericentrin and γ-tubulin are two centrosomal proteins that are present in mouse oocytes and whose localization is similar to that we observed for p-MARCKS (Carabatsos et al., 2000; Meng et al., 2004).

To determine if p-MARCKS is a novel centrosome component, we analyzed oocytes and eggs for co-localization of p-MARCKS with pericentrin and γ-tubulin. Results of these studies revealed a clear co-localization of p-MARCKS with pericentrin (Fig. 5) and γ-tubulin (data not shown). Shortly after GVBD, pericentrin and p-MARCKS co-localized in aggregates surrounding the condensing chromosomes and cytoplasmic foci. In MI and MII eggs, pericentrin and p-MARCKS co-localized at both spindle poles.

During the course of these experiments, we noted a distinct asymmetry between the spindle poles that was previously reported for cells stained for pericentrin (Carabatsos et al., 2000). Prior to rotation of the MI spindle, the appearance of p-MARCKS at the spindle poles was symmetrical and punctate in 29% (20/70) of eggs examined. In the remaining eggs, the p-MARCKS staining was asymmetric such that one pole appeared punctate while the other pole appeared ring-shaped (Figs. 6A and B). In those eggs in which both poles of the MI spindle were clearly observed during/following rotation,

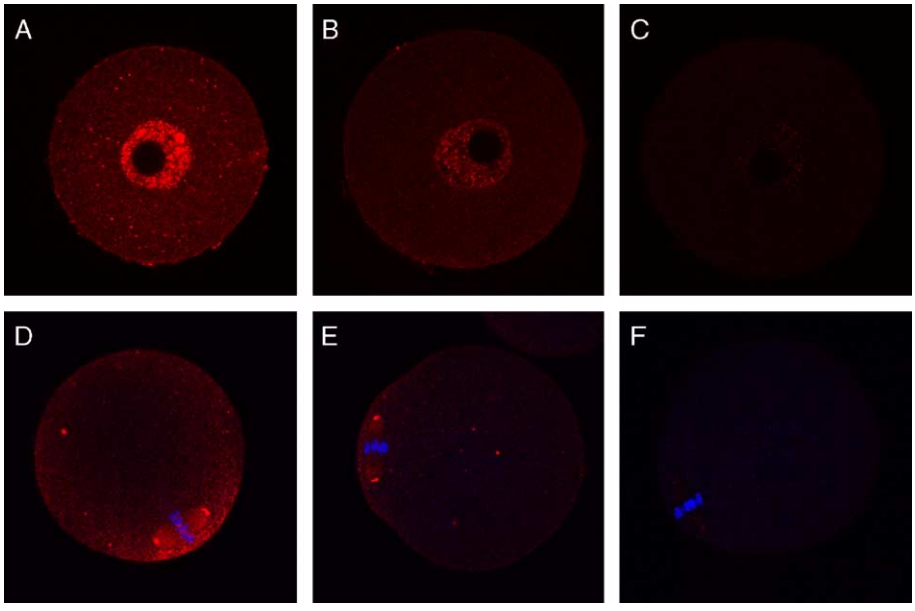

Fig. 3. Effect of blocking peptides on p-MARCKS immunostaining. GV-intact oocytes (A–C) and MII eggs (D–F) were stained with the p-MARCKS antibody alone (A and D) or in the presence of a non-phosphorylated (B and E) or a phosphorylated blocking peptide (C and F). Red, p-MARCKS; blue, DNA. Note that although there was a slight decrease in the signal when the non-phosphorylated blocking peptide was used, the signal was significantly reduced when the phosphorylated blocking peptide was used.

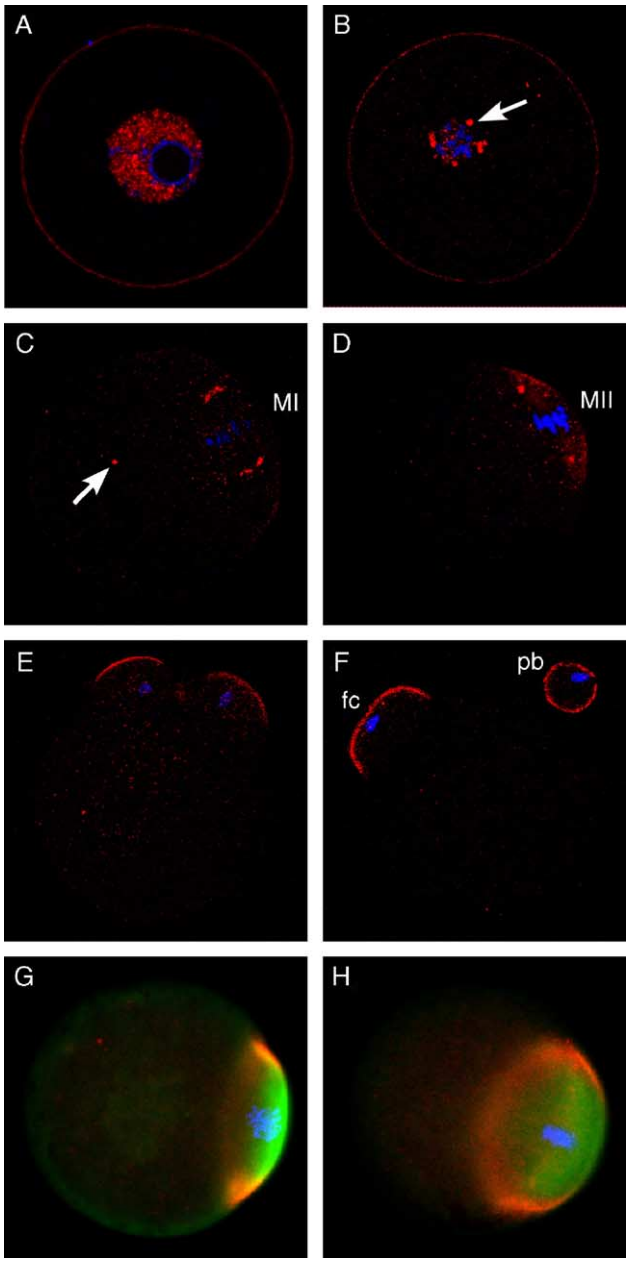

Fig. 4. p-MARCKS localization during meiotic maturation and following fertilization. Cells were immunostained for p-MARCKS (red) and DNA (blue). (A) GV-intact oocyte, (B) GVBD, (C) MI, (D) MII, (E and F) two different 1-cell embryos. Egg chromatin is shown in E, and the first polar body (pb) and sperm chromatin in the fertilization cone (fc) are shown in F. In B and C, the arrows indicate MTOC-like structures. G and H show surface views to highlight the ring-shaped subdomain of p-MARCKS in two MII eggs stained for p-MARCKS (red), DNA (blue) and actin (green). Note in H the punctate p-MARCKS staining at the spindle poles.

p-MARCKS appearance was asymmetric all cases, and in 92% (11/12) of the eggs, the punctate staining pole was oriented towards the cortex, whereas the ring-shaped pole projected centrally (Fig. 6C). Identical staining patterns were observed in cells co-stained for γ-tubulin and p-MARCKS (data not shown). At telophase of meiosis I, p-MARCKS staining was reduced at the spindle poles and enriched at the central region of the spindle (Fig. 6D). Further support for p-MARCKS being a centrosome component is that it localized to regions from which microtubules emanated (Fig. 7).

p-MARCKS remains associated with the cortical region after actin microfilament disruption

Although phosphorylation of MARCKS inhibits its F-actin crosslinking activity, phosphorylation reduces but does not prevent F-actin binding (Hartwig et al., 1992). The localization of p-MARCKS to cortical actin-rich regions in the GV-intact oocyte, MII egg, and 1-cell embryos suggested an interaction between p-MARCKS and microfilaments. To test this idea, the effect of disrupting microfilaments in oocytes and eggs on p-MARCKS localization was assessed.

When oocyte cortical F-actin was completely disrupted following Lat A treatment (10 μM for 10 min), there was no apparent change in p-MARCKS staining (data not shown). When cortical actin was similarly disrupted in MII eggs, p-MARCKS staining became more diffuse and extended into the central region over the MII spindle and regions away from the MII spindle, while spindle pole staining was unchanged (Fig. 8C,D). When microfilaments were permitted to reform by washing out the Lat A and culturing for 4 h, p-MARCKS again localized to the peripheral subdomain in the newly formed cortical actin cap (Fig. 8E,F). Furthermore, as in untreated MII eggs, p-MARCKS did not co-localize with microfilaments that assembled outside the region of the cortical actin cap. An alternative method to disrupt the cortical actin cap is to disrupt spindle microtubules that results in scattering of chromosomes that are then capable of inducing an ectopic cortical actin cap if the chromosome becomes located near the cortex (Evans et al., 2000). Treating eggs for 4 h with nocodazole (10 μM) resulted in formation of multiple ring-shaped p-MARCKS subdomains overlying these dispersed chromosomes (Fig. 9). Taken together, these results suggest that cortical localization of p-MARCKS does not depend on intact microfilaments but that its regional restriction to the periphery of the actin cap does.

p-MARCKS delimits the cortical region involved in polar body extrusion

The peripheral ring-shaped localization of p-MARCKS in MI and MII eggs suggests it may delimit the future region of the abscission ring involved in polar body emission. To test this proposal, MII eggs were activated by incubating them in calcium-free medium containing 10 mM $SrCl_2$ for 1.5 h (Bos-Mikich et al., 1995); this treatment results in a relatively synchronous exit from metaphase II arrest. Egg activation resulted in spindle rotation (Figs. 10A, D, G). Concurrent with spindle

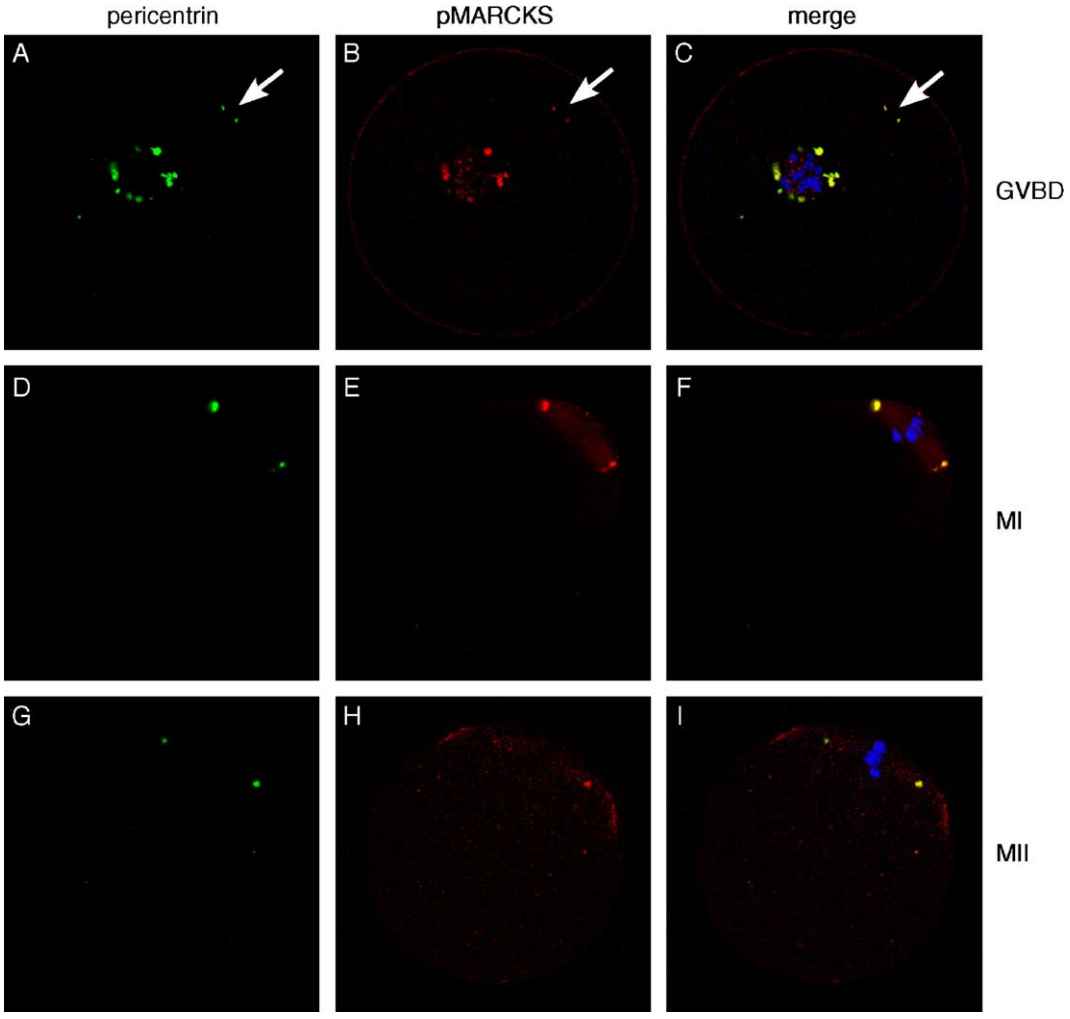

Fig. 5. Co-localization of p-MARCKS and pericentrin during oocyte maturation. Maturing oocytes were stained for pericentrin (green), p-MARCKS (red) and DNA (blue). The arrows indicate cytoplasmic MTOCs based on pericentrin staining.

rotation, cortical p-MARCKS became tightly restricted to the region of the forming abscission ring (Figs. 10B, E, H). Furthermore, p-MARCKS staining at telophase was enriched in the spindle center (Fig. 10E), but at the ends of the spindle was less intense and co-localized with the telophase chromosomes (Fig. 10F).

Phospho-PKC ζ/λ localization during meiotic maturation

To determine if MARCKS is phosphorylated by either a conventional or novel PKC isoform, we treated oocytes and MII-arrested eggs with a biologically active phorbol ester under conditions that inhibit oocyte maturation (Bornslaeger et al., 1986). We observed no increase in the p-MARCKS signal by immunostaining (data not shown), suggesting that an atypical PKC (aPKC; ζ and λ isoforms) isoform phosphorylated MARCKS. Accordingly, we examined the localization of p-PKC ζ/λ during meiotic maturation.

Phospho-PKC ζ/λ was noted in the oocyte's cytoplasm but variable amounts of staining were observed in the GV. The majority of oocytes (81%) had no apparent staining in the GV, whereas 4% had an intense signal and 15% displayed a staining intensity comparable to that observed in the cytoplasm (Figs. 11A–C). Following GVBD, p-PKC ζ/λ became concentrated in aggregates in the region of the condensing chromosomes (Fig. 11D). At MI and MII, p-PKC ζ/λ was localized at the spindle poles and as discrete foci in the cytoplasm (Figs. 11E and F). In the 1-cell embryo, p-PKC ζ/λ localized to both the cytoplasm and pronuclei (Fig. 11G). Staining was specific because incubating the antibody with a specific blocking peptide abolished the signal (Figs. 11H and I). Except for the cortical staining of p-MARCKS, p-PKC ζ/λ staining was essentially identical to that of p-MARCKS. For example, p-PKC ζ/λ was non-uniformly distributed on the MI spindle poles and uniformly distributed on the MII spindle poles.

Discussion

We report here that although MARCKS is widely distributed in mouse oocytes with no obvious change in

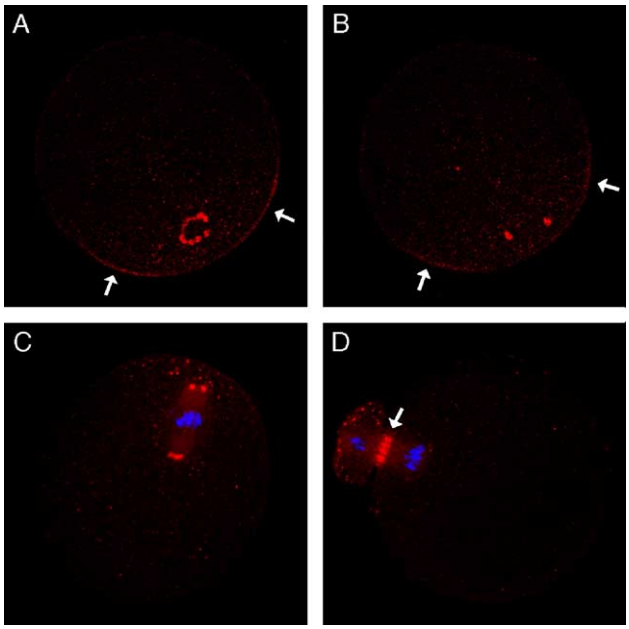

Fig. 6. Asymmetric nature of p-MARCKS on the MI spindle poles. A and B are different confocal planes of the same egg. These panels also show the cortical localization of p-MARCKS (arrows), which is not apparent in the focal plane shown in Figs. 4C and 5E. Panel C shows a fully rotated MI spindle in which the punctate p-MARCKS staining pole apposes the cortex and the ring-shaped pole is directed centrally. Panel D shows an egg in telophase of meiosis I and the arrow indicates p-MARCKS in the central region of the spindle.

its localization during maturation, p-MARCKS is found in previously unreported subcellular locations, including the nucleus of the oocyte, a subdomain of the cortical actin cap, and as a novel centrosome component. The diverse nature of these subcellular locations suggests that p-MARCKS has multiple distinct functions in oocytes and during maturation.

p-MARCKS is clearly localized to the nucleus in the oocyte. To our knowledge, such a nuclear localization has not been reported for other cell types. Note that intense cytoplasmic staining might obscure nuclear staining if standard epifluorescence microscopy was used with the antibody that recognizes total MARCKS. Thus, nuclear staining might be detected in other cell types, especially if the p-MARCKS antibody were used. The presence of a putative bipartite nuclear localization signal in the PSD of MARCKS is consistent with some MARCKS in the nucleus. In fact, a motif similar to the PSD in MARCKS is responsible for the nuclear localization of diacylglycerol kinase-ζ (Topham et al., 1998).

The ability of MARCKS (but not p-MARCKS) to bind to phosphatidylinositol-4,5-bisphosphate (PIP_2) allows MARCKS to modulate PIP_2 accessibility and thereby control the ability of PLC to generate IP_3 and DAG. Regulating generation of these second messengers would influence changes in intracellular Ca^{2+} and PKC activity, respectively. Nuclear lipid signaling is now widely recognized and thought to regulate the cell cycle (Irvine, 2003), though the molecular mechanism(s) are poorly understood. Phosphorylation of MARCKS in the oocyte's nucleus may permit access of nuclear PLC to the released PIP_2 and thereby influence resumption of meiosis. It is of interest that oocyte PLC-β1 translocates to the GV prior to GVBD and is associated with changes in intranuclear Ca^{2+} (Avazeri et al., 2000, 2003). Following fertilization, a series of Ca^{2+} oscillations is initiated by a sperm-derived phospholipase C, PLC-ζ (Knott et al., 2005; Saunders et al., 2002), and terminates with PN formation and PLC-ζ sequestration to the PN (Larman et al., 2004; Marangos et al., 2003; Yoda et al., 2004). Although these findings provide an explanation for cessation of cytoplasmic Ca^{2+} oscillations, why nuclear Ca^{2+} oscillations are not observed is puzzling. The presence of MARCKS in the PN, but not p-MARCKS, and its ability to sequester PIP_2 provide an explanation for the lack of nuclear Ca^{2+} oscillations in the 1-cell embryo.

We find that p-MARCKS is a novel centrosome component, based on its co-localization with pericentrin and γ-tubulin. Centrosomes are the largest non-membranous organelles in most cells and are comprised of many proteins. Pericentrin and γ-tubulin are involved in centrosome organization and microtubule nucleation and are important for spindle integrity (Doxsey et al., 1994). p-MARCKS association with these centrosomal proteins is not lost when microtubules are disrupted in metaphase II eggs (unpublished observations), suggesting p-MARCKS is a component of the microtubule-nucleating complex. Further evidence for this co-localization is the presence of intense

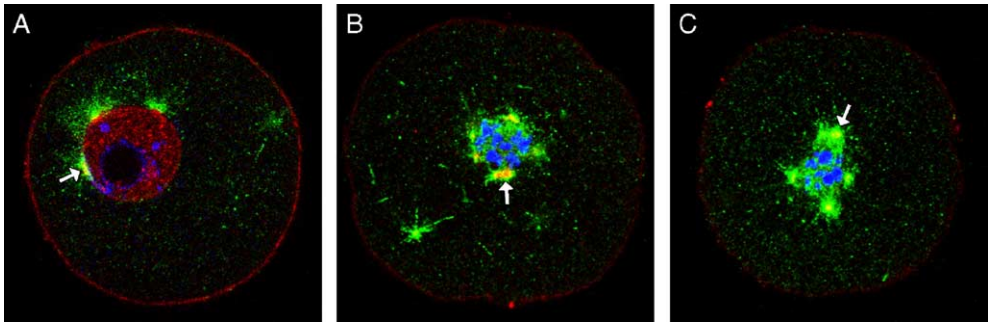

Fig. 7. Co-localization of p-MARCKS and β-tubulin during GVBD. Maturation of GV-intact oocytes was initiated and after 1 h (A) and 2 h (B and C) the cells were stained for p-MARCKS (red), β-tubulin (green), and DNA (blue). Panels B and C show different oocytes. The arrows point to regions of p-MARCKS staining within MTOCs.

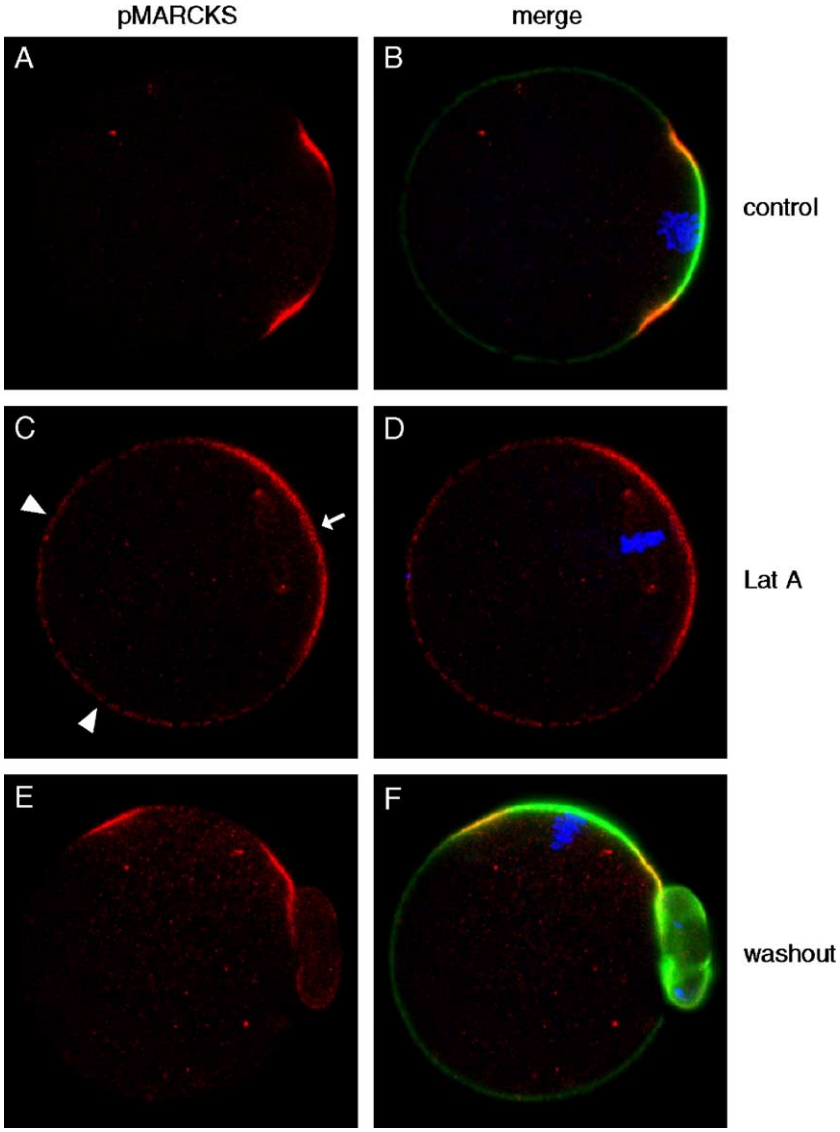

Fig. 8. Association of p-MARCKS with cortical actin. MII eggs were cultured without (A, B) or with Lat A (C–F). Lat A was removed and the eggs cultured for 2 h prior to staining in E and F. p-MARCKS (red), actin (green), and DNA (blue). In panel C, the arrow indicates p-MARCKS staining in the central subdomain over the MII spindle and the arrowheads point to p-MARCKS staining of the cortex not overlying the MII spindle.

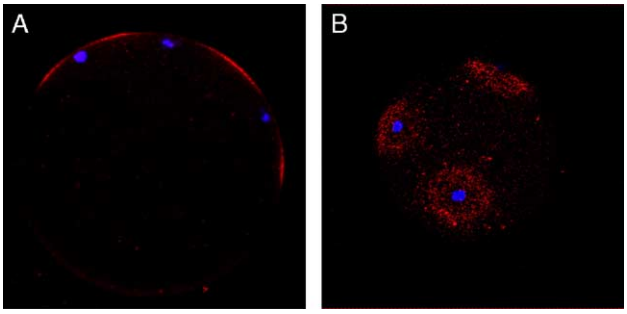

Fig. 9. p-MARCKS staining of MII eggs treated with nocodazole to scatter chromatin. (A) Confocal plane in region of equator showing distinct regions of p-MARCKS enriched around, but not overlying, chromatin. (B) Surface view showing the ring-shaped subdomain of p-MARCKS in the cortex around chromatin. Panels A and B show two different eggs.

foci in the GV apposed to the nucleolus that is reminiscent of a similar staining pattern reported for γ-tubulin (Combelles and Albertini, 2001).

Like pericentrin and γ-tubulin, p-MARCKS is asymmetrically distributed on the MI spindle such that on one pole it appears as punctate loci, whereas at the other pole it appears ring-shaped. This asymmetry apparently develops following spindle migration because 29% of cortically located MI spindles are symmetrical with respect to p-MARCKS appearance. Of particular interest is that the MI spindle preferentially rotates such that the punctate pole apposes the cortex as a prelude to emission of the first polar body. Although the significance of this is unclear, it may relate to the phenomenon of meiotic drive in which specific pairs of chromosomes are preferentially retained in the egg during meiosis I (Agulnik et al., 1990; LeMaire-Adkins and

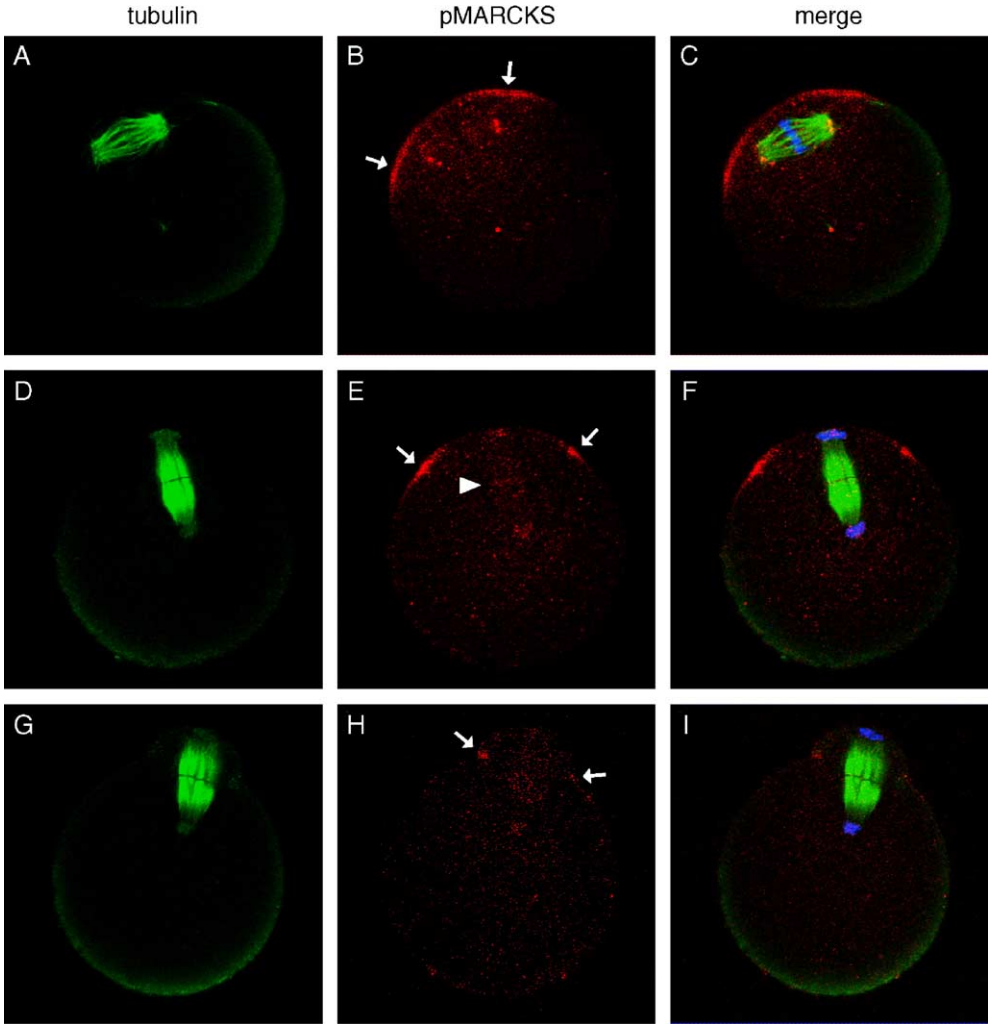

Fig. 10. p-MARCKS delimits the cortical region involved in the second polar body extrusion in association with condensed chromosomes. MII eggs were parthenogenetically activated with $SrCl_2$ and stained for β-tubulin (green), p-MARCKS (red) and DNA (blue). A–C, MII egg; D–F, early telophase; G–I, late telophase. p-MARCKS staining at the cortex (arrows) and central region of the spindle (arrowhead) is shown in panels B, E, and H.

Hunt, 2000). In MII eggs, this asymmetry is no longer observed, suggesting that the asymmetry is not required for the highly asymmetric cell division that constitutes second polar body formation.

p-MARCKS is associated with regions undergoing remodeling of F-actin in the unfertilized and fertilized egg. One of these is the fertilization cone that is induced by sperm chromatin in the cortex and facilitates incorporation of the sperm into the egg following plasma membrane fusion. This transient structure is highly enriched in p-MARCKS, which can bind but not crosslink F-actin (Sundaram et al., 2004), suggesting that MARCKS phosphorylation has a role in the F-actin remodeling process in the fertilization cone. The loss of MARCKS crosslinking function in this region would permit microfilament sliding required for sperm incorporation (McAvey et al., 2002).

We also find that p-MARCKS becomes localized in a subdomain of the cortical actin cap that overlies the MI and MII spindles. Formation of the actin cap, which is larger than the fertilization cone and extends beyond the spindle poles, is also initiated by chromatin in the cortex and characterized by significant thickening of cortical actin. Until now, there has been no indication that subdomains exist within the actin cap. The presence of p-MARCKS delimiting the circumference of the cap and the restricted localization of PAR-3 to the central region (see accompanying paper) define novel subdomains. As discussed in the accompanying paper, the central positioning of PAR-3 and its ability to interact with spindle microtubules and chromatin could tether the spindle to the cortex during spindle rotation and chromosome segregation that occur during MI and MII. The circumferential positioning of p-MARCKS and its lack of F-actin crosslinking activity could facilitate F-actin sliding required for polar body abscission.

The restricted localization of p-MARCKS and PAR-3, which is phosphorylated during oocyte maturation and is a PKC substrate (see accompanying paper), implies that similarly localized PKC isoforms catalyze their phosphorylation. PKCs are classified according to their cofactor requirements (Ron and Kazanietz, 1999). Classical PKCs

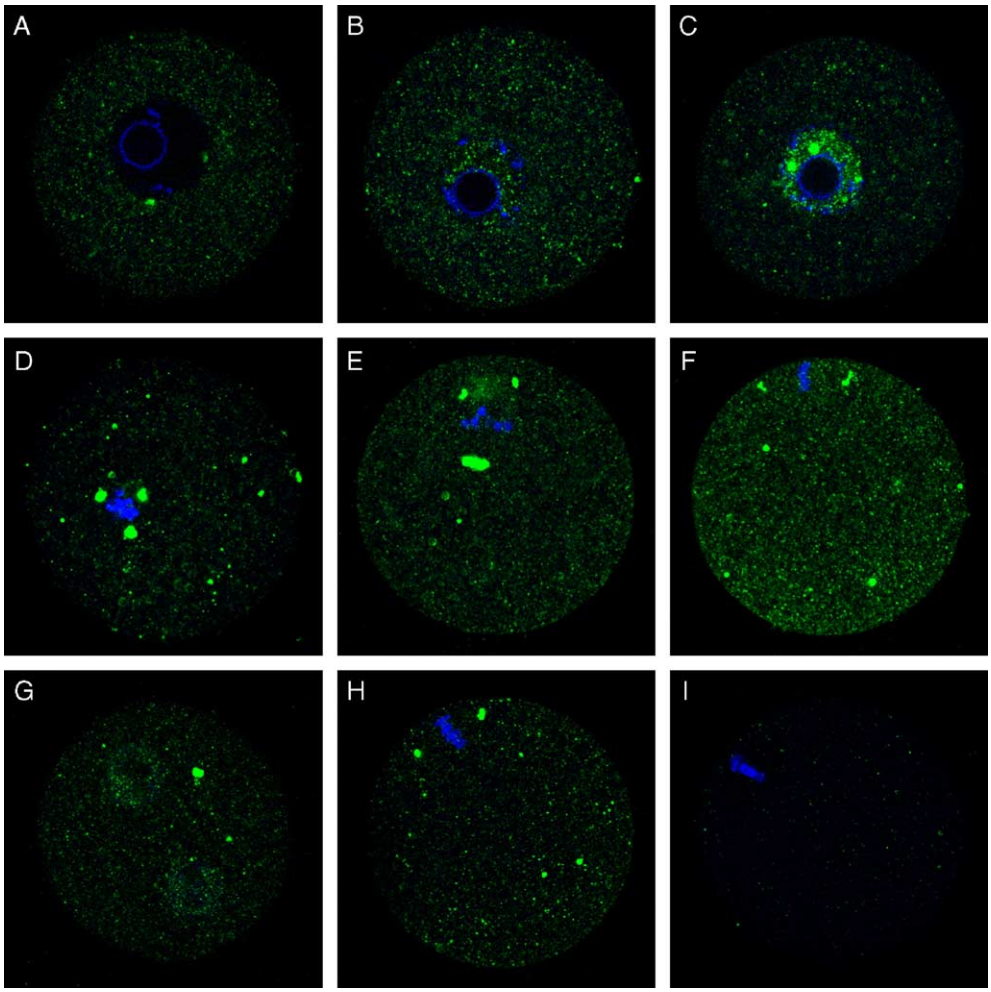

Fig. 11. Phospho-PKC ζ/λ localization during oocyte maturation and in 1-cell embryos. (A–C) Different staining patterns in GV oocytes; (D) GVBD; (E) MI; (F) MII; and (G) 1-cell embryo. The images in panels A–G are representative and can be directly compared because they were processed identically. Antibody specificity was established by staining MII eggs in the absence (H) or presence (I) of a specific blocking peptide. Note that the images shown in H and I cannot be directly compared to those in A–G because they are from a separate experiment.

(α, βI, βII, and γ) are Ca^{+2}- and diacylglycerol-dependent. Novel PKCs (δ, ε, η, μ, and θ) are Ca^{+2} independent but require diacylglycerol for activation, whereas aPKCs (ζ and λ) are neither Ca^{+2}- nor diacylglycerol-dependent. In fact, specific isoforms are localized to specific regions in the maturing oocyte, for example, PKC-δ4 is enriched on the meiotic spindle (Viveiros et al., 2003). Consistent with a previous report (Page Baluch et al., 2004), we also find p-PKC ζ/λ localized at the MII spindle poles, but extended this observation to earlier times during maturation. The similarity in staining patterns for p-PKC ζ/λ and p-MARCKS throughout oocyte maturation is consistent with p-MARCKS being a substrate for p-PKC ζ/λ. In fact, p-PKC ζ/λ may be responsible for the bulk of MARCKS phosphorylation because treatment of oocytes or MII eggs with an activator of conventional and novel PKC isoforms does not result in an increase in p-MARCKS immunostaining signal. Attempts to clarify this issue by inhibiting aPKC using a myristoylated pseudo-substrate (Standaert et al., 1999) were unsuccessful because non-toxic concentrations did not inhibit the autophosphorylation of aPKC and had no effect on p-aPKC localization during maturation (unpublished observations).

There has been rekindled interest in whether or not there is a relationship between egg asymmetry, early axis determination and future cell lineage in the preimplantation mouse embryo. The results presented here indicate that the egg is even more polarized than previously appreciated. A significant amount of p-MARCKS and PAR-3 is lost from the egg proper with second polar body emission. Nevertheless, if the site of polar body emission has a role in establishing the early embryonic axes, some of the residual p-MARCKS and PAR-3 in the region of the abscission site could participate in axis formation.

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