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Protein kinase C delta (PKCδ) interacts with microtubule organizing center (MTOC)-associated proteins and participates in meiotic spindle organization

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ABSTRACT

Defects in meiotic spindle structure can lead to chromosome segregation errors and genomic instability. In this study the potential role of protein kinase C delta (PKCδ) on meiotic spindle organization was evaluated in mouse oocytes. PKCδ was previously shown to be phosphorylated during meiotic maturation and concentrate on the meiotic spindle during metaphases I and II. Currently we show that when phosphorylated on Threonine 505 (pPKCδ^{Thr505}), within the activation loop of its C4 domain, PKCδ expression was restricted to the meiotic spindle poles and a few specific cytoplasmic foci. In addition, pPKCδ^{Thr505} co-localized with two key microtubule organizing center (MTOC)-associated proteins, pericentrin and γ-tubulin. An interaction between pPKCδ^{Thr505} and pericentrin as well as γ-tubulin was confirmed by co-immunoprecipitation analysis using both fetal fibroblast cells and oocytes. Notably, targeted knockdown of PKCδ expression in oocytes using short interfering RNAs effectively reduced pPKCδ^{Thr505} protein expression at MTOCs and leads to a significant ($P < 0.05$) disruption of meiotic spindle organization and chromosome alignment during MI and MII. Moreover, both γ-tubulin and pericentrin expression at MTOCs were decreased in pPKCδ^{Thr505}-depleted oocytes. In sum, these results indicate that pPKCδ^{Thr505} interacts with MTOC-associated proteins and plays a role in meiotic spindle organization in mammalian oocytes.

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Introduction

Microtubule organizing centers (MTOCs), which control the formation and anchorage of microtubules, include centrosomes in higher eukaryotes and spindle pole bodies in fungal organisms. Centrosomes in mammalian somatic cells typically consist of an orthogonal pair of centrioles, surrounded by pericentriolar material (PCM) from where microtubules nucleate. At the molecular level, microtubule nucleation is initiated from ring complexes (γ-TuRC) of γ-tubulin and associated complex proteins (Moritz et al., 1995; Zheng et al., 1995; Raynaud-Messina and Merdes, 2007). These complexes bind dimers of α- and β-tubulins that assemble into profilaments (Moritz and Agard, 2001). Microtubules form at the poles and their growing plus ends are directed outward for chromosome attachment, while the minus ends are anchored at the site of nucleation. The specific targeting of γTuRCs to MTOCs has been attributed to the γ-tubulin associated protein GCP-WD (Nedd1), as its depletion by siRNA results in nearly total loss of γ-TuRCs from MTOCs and lack of microtubule nucleation (Haren et al., 2006; Luders et al., 2006). In turn, the anchoring of γ-TuRCs at MTOCs is mediated, at least in part, by an interaction with pericentrin, a key scaffolding protein onto which many regulatory proteins bind (Doxsey et al., 1994; Zimmerman et al., 2004). Disruption of either γ-tubulin or pericentrin expression in vitro promotes spindle microtubule defects and chromosome segregation errors (Zimmerman et al., 2004). Moreover, targeted deletion of γ-tubulin in mice leads to embryonic loss by the blastocyst stage (Yuba-Kubo et al., 2005).

Meiotic spindle formation in female gametes of various species, including mammals, differs markedly from mitotic spindles in somatic cells. Live imaging analysis in *Drosophila* demonstrated that in contrast to microtubule nucleation from two centrosomes, oocyte spindle microtubules initially nucleate in proximity to the condensing chromosomes. These microtubules subsequently organize and focus into opposing poles to form a bipolar spindle (Matthies et al., 1996; Skold et al., 2005). This mode of spindle assembly has also been observed in mammalian somatic cells following laser ablation of the centrosomes (Khodjakov et al., 2000), and is dependent on the activity of the small GTPase, Ran, as well as the guanine nucleotide exchange factor, RCC1 (Hutchins et al., 2004; Kalab et al., 2006). Studies suggest that meiotic spindle formation in mammalian oocytes may involve similar processes (Dumont et al., 2007). In prophase I arrested (GV-intact) oocytes, normally one to three distinct γ-tubulin foci are detected in the oocyte cytoplasm and eventually localize near the nuclear membrane (Combelles and Albertini, 2001; Can et al., 2003). Upon the resumption of meiosis, the interphase microtubule network within the cytoplasm disassembles and multiple foci of γ-tubulin concentrate near the condensing chromosomes (Combelles and Albertini, 2001). Microtubules nucleate around the chromosomes in a seemingly random manner, but gradually organize to form a barrel-shaped, bipolar, metaphase I spindle. Notably, mammalian oocytes

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contain unique MTOCs, composed of pericentriolar matrix proteins (PCM) including γ-tubulin and pericentrin (Gueth-Hallonet et al., 1993; Doxsey et al., 1994; Calarco, 2000; Carabatsos et al., 2000; Combelles and Albertini, 2001), but lack centrioles (Szollosi et al., 1972; Schatten, 1994). Hence, oocyte MTOCs are often defined as aggregates, or foci, of pericentriolar material that are functionally able to nucleate microtubules. Recent elegant live cell imaging studies have highlighted the dynamic nature of mouse oocyte MTOCs and revealed that MTOCs form de novo from the cytoplasmic microtubule network. Importantly, organized MTOCs in the oocyte function similarly to centrosomes and are essential for meiotic spindle assembly (Schuh and Ellenberg, 2007). Although significant research has focused on understanding centrosome function in mitotic cells, little is known regarding the molecular composition of the unique MTOCs and regulatory proteins that control meiotic spindle formation in mammalian oocytes.

Studies in somatic cells have suggested that protein kinase C (PKC) can regulate microtubule organization (Kiley and Parker, 1995; Jaken and Parker, 2000). PKC proteins are evolutionarily conserved and expressed in diverse organisms. The mammalian protein family consists of 10 serine/threonine kinases classified into three groups based on sequence homology as well as activator and cofactor requirements, which include the 'conventional' (PKCα, βI, βII and -γ), 'novel' (PKCδ, -ε, θ, and -η) and 'atypical' (PKCλ/ι and -ζ) isoforms (Ohno and Nishizuka, 2002—for review). Distinct subcellular distribution patterns of individual PKCs suggest unique isoform-specific functions (Livneh and Fishman, 1997; Black, 2000). Interestingly, several PKCs localize to mitotic spindle microtubules and/or spindle poles, including PKCζ (Lehrich and Forrest, 1994; Liu et al., 2006a), PKCθ (Passalacqua et al., 1999) and PKCβII (Chen et al., 2004). Notably, PKCβII is anchored to centrosomes at the spindle poles by pericentrin binding. Disruption of this interaction leads to loss of microtubule organization and spindle function, aneuploidy and defects in cytokinesis (Chen et al., 2004). The single PKC expressed in *Saccharomyces cerivisiae*, Pkc1p, also associates with the mitotic spindle via its C2 domain (Denis and Cyert, 2005), suggesting some possible conservation of function.

In previous studies, we demonstrated a link between PKC activity in oocytes and the capacity to exit MI (Viveiros et al., 2001, 2004). Our studies focused on PKCδ and demonstrated the expression of the full-length protein as well as a truncated 47kDa carboxy-terminal (catalytic domain) fragment in mouse oocytes, both of which became phosphorylated upon the resumption of meiosis through to MII (Viveiros et al., 2003). By contrast, the unphosphorylated protein predominated shortly after in vitro fertilization and parthenogenetic egg activation. Thus, unphosphorylated PKCδ is characteristic of interphase, while a phosphorylated form is maintained during metaphase. A similar pattern was observed during early embryonic mitotic divisions, confirming that this kinase is phosphorylated in a cell cycle dependent manner. Immunofluorescence analysis revealed diffuse PKCδ expression in the oocyte cytoplasm during meiotic maturation. Intriguingly, the protein also concentrated on meiotic spindle microtubules during the metaphase I to anaphase transition (Viveiros et al., 2001, 2003). PKCδ association with the second meiotic spindle following egg activation to promote anaphase II has also been demonstrated (Tatone et al., 2003; Baluch et al., 2004). We, therefore, sought to assess the expression and function of phosphorylated PKCδ in mouse oocytes. PKC proteins are known to undergo a series of ordered 'priming' phosphorylations that regulate enzyme activity and stability (Dutil et al., 1998; Le Good et al., 1998, Newton, 2003). An initial phosphorylation occurs on a key threonine residue within the activation loop of the C4 domain, which corresponds to Threonine 505 (Thr505) in mouse PKCδ. In this study, we provide evidence that PKCδ phosphorylated on Thr505 (denoted as pPKCδ^{Thr505}) is expressed upon meiotic resumption in oocytes. Notably, this kinase targets to oocyte MTOCs and interacts with key MTOC-associated proteins. Moreover, loss of pPKCδ^{Thr505} expression in oocytes significantly disrupts meiotic spindle organization.

Materials and methods

Oocyte collection and culture

The temporal and spatial distribution patterns of pPKCδ^{Thr505} were assessed in fully-grown oocytes during progressive stages of meiotic maturation. Oocytes were recovered from B6D2F1, (C57BL/6J x DBA/2J F1) mice. Females, 21 to 23-day old, were injected with 5IU PMSG (EMD Biosciences, San Diego, CA) to stimulate pre-ovulatory follicle development, and cumulus cell-oocyte complexes (COCs) were isolated from the ovaries 44 to 48h later. GV-intact oocytes were immediately isolated from the recovered ovaries. Oocytes undergoing germinal vesicle breakdown (GVB), as well as those at prometaphase and the MI stage were collected following a 2, 4 and 8-hour culture, respectively. Mature MII eggs were collected from the oviducts of superovulated females, approximately 16h after treatment with 5IU hCG (EMD Biosciences). Oocytes were cultured in Minimal Essential Medium (MEM) supplemented with 3mg/ml crystallized bovine serum albumin (BSA, Sigma, St. Louis, MO) and 5% fetal calf serum (Hyclone). Prophase I arrest was maintained by culturing GV-intact oocytes with 2.1μg/ml of the phosphodiesterase inhibitor, milrinone (Sigma). All cultures were maintained at 37°C with 5% CO_2, 5% O_2 and 90% N_2.

Immunofluorescence analysis

Oocytes were fixed with 4% paraformaldehyde in PEM Buffer (100mM Pipes, pH 6.9, 1mM MgCl2, 1mM EGTA) with 0.5% Triton-X for 1h, then rinsed and blocked in PBS with 5% serum. A primary antibody directed against PKCδ phosphorylated on Thr507 (Santa Cruz Biotechnology, Santa Cruz, CA) was used, which corresponds to Thr505 in mouse (subsequently referred to as anti-pPKCδ^{Thr505}). Additional antibodies were used to detect acetylated tubulin (Sigma), γ-tubulin (Sigma), pericentrin (BD Biosciences, San Jose, CA), and phosphorylated MEK1/2 (pMEK, Santa Cruz). Anti-mouse and anti-rabbit Alexa Fluor conjugated 488 and 555 secondary antibodies were purchased from Molecular Probes (Invitrogen). DNA was detected with DAPI (Sigma). Fluorescence was assessed using an upright fluorescent microscope (Leica Microsystems).

Western blotting

Samples of denuded GV, MI and MII-stage oocytes were collected and frozen in Laemmli buffer with protease inhibitors (Viveiros et al., 2003). Prior to analysis the samples were thawed and subsequently heated to 100c°C for 5min. The proteins were separated in 10% acrylamide gels containing 0.1% SDS, then transferred onto hydrophobic PVDF membranes (Amersham, Piscataway, NJ). Membranes were blocked (PBS supplemented with 2% BSA and 0.1% Tween-20) overnight at 4°C, then incubated with anti-pPKCδ^{Thr505} for 2h at room temperature, followed by three (20-minute) washes in PBST (PBS with 0.1% Tween-20). A peroxidase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) was added for 1 h prior to processing using an ECL-plus detection system (Amersham).

Co-immunoprecipitation

To determine whether endogenous pPKCδ^{Thr505} interacts with specific proteins, co-immunoprecipitation studies were undertaken. Lysates were prepared from MII-stage oocytes (n = 500) collected from superovulated female mice as well as mouse embryonic fibroblast cells (MEFs). The fibroblast cells were cultured in DMEM with 10% fetal calf serum and grown to confluency in 10cm dishes. Analysis was carried out using FroFound™ Mammalian Co-Immunoprecipitation Kit (Pierce, Rockford, IL) in accordance with the manufacturer's instructions. Briefly, rabbit anti-pPKCδ^{Thr505} was dialyzed against PBS, and immobilized on the coupling gel. Non-related goat anti-Mad2 was used as an immunoprecipitate control. Cell lysates were prepared, immediately transferred onto the antibody-coupled gel, and incubated for 2h. The co-immunoprecipitation complex was eluted and processed for SDS-PAGE analysis. The immune-complex samples were dissolved in sample buffer, resolved on 10% SDS gel, and analyzed by Western blotting. Following protein transfer, the membranes were blocked in TBST with 5% BSA over night at 4°C, then probed with anti-γ-tubulin, anti-pericentrin or anti-β-tubulin.

Knockdown of pPKCδ^{Thr505} expression in oocytes by siRNA

To assess possible function, short interfering RNAs (siRNA), approximately 20–25 nucleotides in length, specific for PKCδ were used to knockdown transcript levels in oocytes. Control groups included (i) non-injected oocytes that were subject to the same culture conditions and (ii) oocytes injected with siRNAs specific for a different PKC isoform, PKCγ. For each group, 10pl of a 10μM siRNA solution (Santa Cruz) was microinjected directly into the cytoplasm of denuded, fully-grown, oocytes arrested at prophase I in medium supplemented with 2.1μg/ml milrinone. Sterile Femtotip capillaries and a Femptojet microinjector (Eppendorf, Westbury, NY) were used to standardize injection volumes. Following oocyte microinjection, GV-arrest was maintained for 24h. The oocytes were subsequently washed, transferred to fresh medium and cultured for an additional 17h. Initial control experiments were

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