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Inhibition of protein kinase C ζ blocks the attachment of stable microtubules to kinetochores leading to abnormal chromosome alignment

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Abstract

The attachment of spindle microtubules to kinetochores is crucial for accurate segregation of chromosomes to daughter cells during mitosis. While a growing number of proteins involving this step are being identified, its molecular mechanisms are still not clear. Here we show that protein kinase C ζ (PKCζ) is localized at the mitotic spindle during mitosis and plays a role in stable kinetochore-microtubule attachment. Striking staining for PKCζ was observed at the mitotic spindle and spindle poles in cells at prometaphase and metaphase. PKCζ molecules at these stages were phosphorylated at Thr-410, as detected by a phosphospecific antibody. PKCζ was also detected at the spindle midzone and the midbody during anaphase and telophase, respectively, and PKCζ at these stages was no longer phosphorylated at Thr-410. The polarity determinants Par3 and Par6, which are known to associate with PKCζ, were also localized to the spindles and spindle poles at prometaphase and metaphase. Knockdown of PKCζ by RNA interference affected normal chromosome alignment leading to generation of cells with aberrant nuclei. A specific PKCζ inhibitor strongly blocked the formation of cold-sensitive stable kinetochore microtubules, and thus prevented microtubule-kinetochore attachment. Treatment of cells with the PKCζ inhibitor also dislocated the minus-end directed motor protein dynein from kinetochores, but not the mitotic checkpoint proteins Mad2 and CENP-E. Prolonged exposure to the PKCζ inhibitor eventually resulted in cell death. These results suggest a critical role of PKCζ in spindle microtubule-kinetochore attachment and subsequent chromosomal separation.

Keywords: Chromosome separation; Mitosis; Par3; Par6; Stable microtubules

1. Introduction

Kinetochores are structures that form the interface between the chromosomes and the microtubules of the mitotic spindle [1–3]. Kinetochores function as the attachment site of the chromosome to the spindle microtubules and monitor the attachment. When abnormal attachment of chromosomes to the spindles is detected, the mitotic checkpoint is activated. Chromosome separation is achieved by the dynamic instability of spindle microtubules, which alternates between phases of growth and shrinkage. The highly dynamic nature of microtubule behavior is integrated with the kinetochore function to move and segregate chromosomes. Many proteins have been identified that localize and function in the kinetochores. These kinetochore-associated proteins include those in chromatid pairing such as Survivin and Aurora B [4,5], those in kinetochore assembly, such as CENP-A, C, G, H [6], and those in regulating microtubule attachment and dynamics as well as checkpoint signaling such as Mad1, Mad2, Bub1, BubR1, dynein and CENP-E [1,7,8].

Cytoplasmic dynein is a large, multisubunit ATPase that moves along microtubules toward their minus-ends [9]. Dynein transports and localizes membranous organelles, and is a transient kinetochore component whose binding is regulated by microtubule attachment [10–12]. Dynein is recruited to kinetochores at prometaphase, but gradually loses the attachment from metaphase to anaphase [13]. Dynein and CENP-E are the only known kinetochore proteins with demonstrated motor activity. Dynein may contribute to kinetochore microtubule capture and chromosome movement [3].

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Protein kinase C (PKC) is involved in intracellular signal transduction, cell proliferation, apoptosis, cell cycle and polarity determination [14,15]. The PKC family consists of 11 different serine/threonine kinases that are divided into three subfamilies depending on their structure similarity and cofactor requirements. The conventional PKCs are diacylglycerol (DAG)-, phospholipids- and calcium-dependent, and include PKC α, βI, βII and γ. Novel PKCs are DAG- and phospholipid-dependent, but calcium-independent, and include PKCε, η, μ, θ and δ. The third group is the atypical PKC (aPKC) isoforms that are both DAG- and calcium-independent, and includes PKCζ and human PKCι/mouse PKCλ. Recently, an isoform with a new PKCζ catalytic domain, designated PKMζ, was described to be specific to the brain [16]. Another newly identified PKCζ member, PKCζII, which is a truncated form of PKCζ, is functionally involved in cell polarity through inhibition of tight junction formation [17].

Most studies on PKCζ functions are focused on the development of polarity in mammalian cells and lower eukaryotic cells [14,15]. Recently, PKCζ has been reported to associate with meiotic spindles. Inhibition of PKCζ, but not other PKC isoforms results in rapid disruption of meiotic spindles in mouse eggs, indicating a role of PKCζ in regulating spindle organization and stability during mouse oocyte meiosis [18,19]. PKCζ is also localized to the mitotic apparatus in primary cell cultures of shark rectal gland and CHO cells [20]. However, the biological functions of PKCζ in mitotic cells are still unknown.

We have previously identified PKCζ as a protein which binds to the guanine nucleotide exchange factor ECT2 [21]. ECT2 regulates cytokinesis in the steps of contractile ring formation and abscission [22,23]. However, ECT2 is localized to the mitotic spindle during mitosis [23,24]. In this report, we show that PKCζ is also localized to the mitotic spindle and the spindle poles in HeLa cells. Knockdown of PKCζ by RNA interference caused abnormal kinetochore attachment to the mitotic spindle in prometaphase. Treatment of cells with an PKCζ-specific inhibitor caused disruption of the mitotic spindle. We propose a new role of PKCζ in spindle attachment to kinetochores in mitosis.

2. Materials and methods

2.1. Antibodies and reagents

Anti-PKCζ (C20; dilution 1:250) and blocking peptides were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-CREST serum (1:800) and Rhodamine-conjugated secondary anti-human antibody (1:100) was purchased from Cortex Biochem (San Leandro, CA). Anti-Mad2 antibody (1:250) was from Covance (Richmond, CA). Anti-Par3 antibodies (1:500 dilution) were from Upstate (Lake Placid, NY) and a gift from S. Ohno [25], and both antibodies gave similar staining patterns. Anti-phospho-PKCζ/λ (Thr-410/403) antibody was purchased from Cell Signaling (Beverly, MA). Anti-CENP-E antibody (1:250) was from abCam (Cambridge, MA). Monoclonal anti-β-tubulin (1:500) and monoclonal anti-dynein (1:500) antibodies were purchased from Sigma (St. Louis, MO). Alex-Flour-conjugated secondary antibody was purchased from Molecular Probes (Eugene, OR).

All the PKC inhibitors were purchased from Calbiochem (La Jolla, CA). The PKCζ pseudosubstrate inhibitor (Cat. # 539624) was derived from amino acid 113–125 of the pseudosubstrate recognition region of PKCζ, and rendered membrane permeable by myristoylation. The broad PKC inhibitor bisindolylmaleimide (Cat. # 203291) is membrane permeable, and acts on the catalytic subunit of PKCα, βI, βII,γ, δ and ε isotypes by blocking the binding of ATP. The other two inhibitors tested were the myristoylated pseudosubstrate of PKCη (Cat. # 539604) and that of PKCα and β (Cat. # 476480). All the pseudosubstrate inhibitors were maintained at 1 mM as stocks and diluted to a working concentration of 20 μM.

2.2. Cell culture

HeLa cells were grown in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified chamber under 5% CO_2 atmosphere at 37 °C. For RNAi analysis, HeLa cells were transfected with SiGenome Smartpool for PKCζ (Cat. M-003526-02, Dharmacon, Lafayette, CO) or control luciferase siRNA GL2 by Oligofectamine (Invitrogen, Carlsbad, CA).

2.3. Immunofluorescence and confocal microscopy

Cells grown on a cover glass were washed with PBS twice, and pretreated with 0.5% Triton X-100 in ice-cold PHEM buffer (60 mM pipes, 25 mM Hepes, 10 mM EGTA and 1 mM Mg-acetate, pH 6.9) for 1 min. After fixation in 4% PFA and quenching in 100 mM glycine for 15 min, cells were extracted with 0.5% Triton X-100 for 10 min, and then blocked with 3% BSA for 30 min. Samples were incubated with primary antibodies in PBS/BSA for 1–2 h followed with Alex-Flour conjugated secondary antibody for an additional hour. Cells were washed in PBS and incubated with DAPI, and then mounted on a glass slide using the Antifade reagent. All images were obtained using the Zeiss LSM 510 confocal microscope. For antibody competition, cells were subjected to staining for PKCζ as described above except for preincubation with 5-fold excess of peptides for 30 min before incubation with primary antibody.

3. Results

3.1. PKCζ is localized to the mitotic spindle and spindle poles during mitosis

To study the biological function of PKCζ in mitosis, we first examined its subcellular localization in mitosis using affinity-purified anti-PKCζ antibody by immunofluorescence microscopy (Fig. 1A). In interphase cells, PKCζ was detected in both the cytoplasm and nucleus. In prometaphase cells, a striking staining for PKCζ was observed at the spindle poles, as detected by colocalization with γ-tubulin (Fig. 1A second row, arrow). In metaphase cells, PKCζ was also detected at the mitotic spindle as well as spindle poles. Localization of PKCζ at the mitotic spindle was also confirmed by colocalization with β-tubulin (Fig. 1B). At anaphase, PKCζ was detected at the central spindle, and strong staining was observed at the spindle midzone (Fig. 1B third row, arrows). During telophase/cytokinesis, PKCζ was recruited to the midbody (Fig. 1B bottom row, arrows). Strong staining was observed at the center of the midbody which was hemmed by two thick bundles of tubulin (Fig. 1B bottom row, inset). Incubation of antibody with blocking peptides efficiently blocked the signal by anti-PKCζ antibody (Supplementary Fig. 1), suggesting that the antibody specifically detects PKCζ. These results suggest that PKCζ is localized at the mitotic spindle and spindle poles.

3.2. PKCζ at the mitotic spindle is phosphorylated at Thr-410, but dephosphorylated before cytokinesis

It is known that PKCζ is activated by phosphorylation at Thr-410 [14]. To test whether PKCζ at the mitotic spindle is