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Chk1 is required for the metaphase–anaphase transition via regulating the expression and localization of Cdc20 and Mad2

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ABSTRACT

Aims: The checkpoint kinase 1 (Chk1) functions not only in genotoxic stresses but also in normal cell cycle progression, particularly in the initiation, progression and fidelity of unperturbed mitosis. In this study, we investigated the role of Chk1 in regulating the metaphase–anaphase transition in mammalian cells.
Main methods: The mitotic progression was monitored by flow cytometry analysis. The levels of cyclin B1, Cdc20 and Mad2 were measured by Western blotting. Metaphase chromosome alignment and the subcellular localization of Cdc20 and Mad2 were analyzed by immunofluorescence and confocal microscopy.
Key findings: Cyclin B1 degradation and the metaphase–anaphase transition were severely blocked by Chk1 siRNA. Depletion of Chk1 induced chromosome alignment defect in metaphase cells. The kinetochore localization of Cdc20, Mad2 was disrupted in Chk1 depleted cells. Chk1 abrogation also dramatically reduced the protein expression levels of Cdc20 and Mad2.
Significance: These results strongly suggest that Chk1 is required for the metaphase–anaphase transition via regulating the subcellular localization and the expression of Cdc20 and Mad2.

Introduction

It is well established that checkpoint kinase 1 (Chk1) is a conserved serine/threonine protein kinase that plays central roles in cell survival, proliferation and multiple checkpoint responses in eukaryotic cells exposed to various genotoxic stresses (Enders, 2008; Peddibhotla and Rosen, 2009; Smith et al., 2010; Yu, 2007; Zachos and Gillespie, 2007). In mammals, Chk1's function is essential for embryonic development as Chk1 knockout mice are embryonic lethal. Chk1 deficient blastocysts and embryonic stem cells show severe proliferation defects and impaired cell cycle checkpoint response (Liu et al., 2000; Takai et al., 2000). However, Chk1 deficient tumor cells can be viable, as was shown with the generation of a somatic avian B-lymphoma DT40 cell line deficient for Chk1 (Zachos et al., 2003). Chk1 −/− DT40 cells exhibit an impaired G2/M arrest, hypersensitive to killing by ionizing radiation and premature mitosis induced by stalled replication forks and the suppression of futile origin firing (Zachos et al., 2003, 2005).

Several lines of evidence demonstrate Chk1 functions not only in genotoxic stresses but also in normal cell cycle progression, particularly in regulating the initiation, progression and fidelity of unperturbed mitosis (Chila et al., 2013; Enomoto et al., 2009; Kramer et al., 2004; Lam et al., 2004; Royou et al., 2008; Tang et al., 2006; Zachos et al., 2007; Zachos and Gillespie, 2007). It has been shown that human Chk1 kinase localizes to centrosomes in the interphase and the centrosome-associated Chk1 prevents premature activation of cyclin B-Cdk1 kinase, which contributes to the proper timing of the initiation of mitosis (Kramer et al., 2004). In *Drosophila*, Grp (Chk1) prevents nuclear cyclin-dependent kinase 1 (Cdk1) activation by delaying cyclin B nuclear accumulation (Royou et al., 2008). Interestingly, Chk1 is a Cdk target, Chk1 can be phosphorylated at serine 286 and serine 301 by Cdk1 during mitosis, the mitotic phosphorylation of Chk1 promotes the translocation of Chk1 from the nucleus to the cytoplasm in prophase, thus releasing an inhibitory effect of Chk1 on Cdk1 to promote mitotic entry (Enomoto et al., 2009). The functional roles of Chk1 in regulating spindle checkpoint were investigated by several studies. Recent studies show that Chk1 is specifically required for the tension-sensing branch of the spindle checkpoint and Chk1 can directly phosphorylate and enhance Aurora B kinase activity (Petsalaki et al., 2011; Zachos et al., 2007). Other report demonstrates that abrogation of Chk1 in mitotic mammalian cells results in cytokinetic regression and binucleation, chromosome lagging and/or non-disjunction and abnormal localization of Aurora B (Peddibhotla et al., 2009). In addition, Chk1 can negatively regulate Plk1 to inactivate the spindle assembly checkpoint and cause chromosome lagging during anaphase (Tang et al., 2006).

Chromosome segregation occurs during the metaphase–anaphase transition and is triggered by a ubiquitin ligase known as the

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anaphase-promoting complex or cyclosome (APC). APC activity is strictly dependent on two co-activator proteins Cdc20 and Cdh1 that associate with APC/C during specific periods of the cell cycle (Peters, 2006). APC–Cdc20 is negatively regulated by spindle assembly checkpoint. During mitosis, spindle fibers attach to the centromeres via the kinetochores. Upon checkpoint activation, unattached kinetochores catalyze the formation of the mitotic checkpoint complex (MCC) composed of BubR1, Bub3, Mad2 and Cdc20, leading to the inhibition of APC (van Leuken et al., 2008; Yu, 2002). Once all the chromosomes are aligned with their kinetochores attached to the spindle, Cdc20 binds to APC and targets the ubiquitylation and degradation of securin and cyclin B1 (Lara-Gonzalez et al., 2012).

Here, we study the role of Chk1 on mitotic progression, particularly the metaphase–anaphase transition in mammalian cells. We show that Chk1 depletion delays cyclin B1 proteolysis and the metaphase–anaphase transition. Chk1 is required for the metaphase chromosome alignment and the kinetochore localization of Cdc20 and Mad2. Abrogation of Chk1 results in reduced expression of Cdc20 and Mad2. Collectively, Chk1 is required for the metaphase–anaphase transition via regulating the localization and expression of spindle checkpoint proteins Cdc20 and Mad2.

Materials and methods

Cell culture and treatment

Human cervical carcinoma HeLa cells and human colon carcinoma BE cells were cultured in Dulbecco's modified Eagle's medium (Gibco/Invitrogen Ltd.) supplemented with 10% fetal bovine serum, 10 U/ml penicillin–streptomycin at 37 °C in a humidified 5% CO_2 incubator. DT40 B-lymphoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% chicken serum, 10^{-5} M β-mercaptoethanol at 37 °C in a humidified 5% CO_2 incubator. To synchronize cells in early mitosis, cells were treated with 50 ng/ml nocodazole (Sigma, St. Louis, MO, USA) for 10–12 h. Mitotic cells were collected by shake-off and washed three times with PBS and then cultured in fresh medium for experiment. To arrest cells in metaphase, cells were incubated for 30–60 min with 25 μM MG132 (Sigma, St. Louis, MO, USA) as described previously (Potapova et al., 2006). To activate spindle checkpoint, wild-type or Chk1 −/− DT40 cells were treated with 10 μM taxol (Sigma, St. Louis, MO, USA) for 0, 5, 10 and 15 h.

Plasmid and siRNA transfection

YFP-Mad2 fusion gene was introduced into HeLa cells by Amaxa Nucleofector kit (Lonza) following manufacturer's protocol. Control siRNA (sequence: 5′-UUCUCCGAACGUGUCACGU-3′) or Chk1 siRNA smart pool (Dharmacon, Thermo Scientific) was transfected into HeLa cells 48 h prior to analysis or treatment with drugs by using oligofectamine reagent (Invitrogen, USA).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature followed by permeabilization with 0.25% Triton-X-100 in PBS for 5 min. Fixed preparations were blocked with 3% BSA in PBS for 30 min at room temperature, then incubated with primary antibodies for 1 h at room temperature. Primary antibodies were: Cdc20 (Cat No. sc-8358, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Aurora B (Cat No. ab2254, Cambridge, Abcam, UK), CENP-B (Cat No. ab25734, Cambridge, Abcam, UK). Secondary antibodies were Alexa Fluor 488 or 555 conjugates (Invitrogen). Images were captured using an Olympus confocal microscope.

Flow cytometry

HeLa cells released from nocodazole block were fixed in 70% ethanol in phosphate-buffered saline (PBS) overnight. To determine mitotic index, fixed cells were incubated with anti-phospho serine 10 histone H3 (pH3) antibody (Cat No. sc-8656, Santa Cruz Biotechnology, Santa Curz, CA, USA) followed by FITC-conjugated secondary antibody (Jackson Labs, Bar Harbor, ME, USA). Cells were then counterstained with propidium iodide (PI) and analyzed for pH3 fluorescence and DNA content by the use of a Beckton Dickinson FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting

Cells were lysed in ice-cold whole-cell extract buffer as described previously for 30 min on ice (Xu et al., 2010). Lysates were cleared by centrifugation at 13,200 rpm for 15 min. Densitometric analysis was performed with Quantity One (Bio-Rad). Cell extracts were resolved by SDS-PAGE, and analyzed by Western blotting. Membranes were probed with the following antibodies: monoclonal antibodies against Chk1 (Cat No. sc-8408) and Cdc20 (Cat No. sc-8358) from Santa Cruz Biotechnology; Monoclonal antibody against cyclin B1 (Cat No. 554179) from BD Biosciences; Polyclonal antibodies against BubR1 (Cat No. A300-386A) and Mad2 (Cat No. A300-301A) were from Bethyl Laboratories. A custom polyclonal antiserum against avian Aurora B was generated by Eurogentec. Briefly, the N-terminus 15 amino acids of avian Aurora B were selected as the immunogen for antibody. The purified immunogen was injected into 2 rabbits to elicit an immune response. Both pre-immune serum and serum from the final bleed post-immunization were tested. The final bleed from one rabbit detects a single band at 38 kDa.

Statistical analysis

All results were expressed as means ± SD of three independent experiments. Statistical analyses were performed using Student's two-tailed t-test. Values of $^*p < 0.05$ were considered to be significant.

Results

Depletion of Chk1 delays cyclin B1 degradation and mitotic progression

One of the key events during the metaphase–anaphase (M/A) transition is the degradation of mitotic B-type cyclins by the anaphase-promoting complex (APC). It has previously been reported that Chk1 is required for mitotic progression and cytokinesis. To examine the role of Chk1 on the metaphase–anaphase transition, we transfected human cervical carcinoma HeLa cells or human colon carcinoma BE cells with Chk1 siRNA pool, followed by nocodazole synchronization. Mitotic cells were collected by shake-off and released to a fresh medium up to 4 h. The dynamic change of cyclin B1 protein levels was detected by Western blot. As shown in Fig. 1A, the amount of intracellular cyclin B1 protein levels decreased dramatically within 1 h after release from the nocodazole block. By contrast, in Chk1 depleted cells, cyclin B1 maintained a relatively high level even at 2 h after nocodazole release, indicating that Chk1 depletion caused a delay of cyclin B1 degradation. We also performed flow cytometry (FACS) to examine mitotic progression in control and Chk1 depleted HeLa cells. The cells were stained with phosphor S10 Histone H3 antibody and propidium iodide (PI) to discriminate the G1 phase and mitotic cells. As shown by FACS analysis in Fig. 1B, abrogation of Chk1 led to a significant delay in mitotic progression compared with control cells. These results indicate that Chk1 depletion interferes with cyclin B1 degradation and subsequent mitotic exit.