Cdc25B Phosphatase Participates in Maintaining Metaphase II Arrest in Mouse Oocytes

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Cdc25B is an essential regulator for meiotic resumption in mouse oocytes. However, the role of this phosphatase during the later stage of the meiotic cell cycle is not known. In this study, we investigated the role of Cdc25B during metaphase II (MII) arrest in mouse oocytes. Cdc25B was extensively phosphorylated during MII arrest with an increase in the phosphatase activity toward Cdk1. Downregulation of Cdc25B by antibody injection induced the formation of a pronucleus-like structure. Conversely, overexpression of Cdc25B inhibited Ca²⁺-mediated release from MII arrest. Moreover, Cdc25B was immediately dephosphorylated and hence inactivated during MII exit, suggesting that Cdk1 phosphorylation is required to exit from MII arrest. Interestingly, this inactivation occurred prior to cyclin B degradation. Taken together, our data demonstrate that MII arrest in mouse oocytes is tightly regulated not only by the proteolytic degradation of cyclin B but also by dynamic phosphorylation of Cdk1.

INTRODUCTION

Mature mammalian oocytes are arrested at the metaphase of second meiosis (MII) by cytosolic factor (CSF) until fertilization. The molecular mechanism that controls this arrest depends on the regulation of M-phase promoting factor (MPF), a complex of Cdk1 and cyclin B. MPF activity is negatively regulated by the phosphorylations of two highly conserved residues of Cdk1, Thr14 and Tyr15 (Lew and Kornbluth, 1996). In mouse oocytes, these inhibitory phosphorylations are catalyzed by the Wee1B kinase (Han et al., 2005), whereas dephosphorylation (and hence activation of Cdk1) is mediated by dual-specificity phosphatase Cdc25B (Lincoln et al., 2002). Although three isoforms of Cdc25s, Cdc25A, Cdc25B, and Cdc25C are expressed in mouse oocytes, Cdc25B has been known to be essential for meiosis resumption (Lincoln et al., 2002). Therefore, it seems likely that MPF activity is regulated by a balance between the activity of the Wee1B kinase and the Cdc25B phosphatase. However, this is only the case for meiotic resumption. After oocytes have resumed meiosis and arrest at the MII stage, MPF activity is regulated by the steady-state level of cyclin B.

Upon fertilization, this steady-state is rapidly disrupted by the anaphase promoting complex/cyclosome (APC/C)-mediated degradation of cyclin B (Jones, 2005). Thus, the current concept is that cyclin B turnover is the dominant mechanism for MPF regulation during MII arrest. However, mounting evidence suggests that Cdk1 phosphorylation also plays a critical role during MII arrest and exit. For instance, the depletion of Wee1B impairs the release from MII arrest (Oh et al., 2011). Moreover, downregulation of Cdc25A activity induces pronuclear formation in mouse oocytes (Oh et al., 2013), supporting the phosphorylation-dependent regulation of MPF during MII arrest. However, there is no direct evidence that Cdc25B is required for MII arrest, because Cdc25B null oocytes are arrested at prophase I stage (Lincoln et al., 2002). Therefore, in this study, we investigated the role of Cdc25B during MII arrest in order to dissect the mechanism regulating Cdk1 phosphorylation in mouse oocytes.

MATERIALS AND METHODS

Animals

All mice used in this study were 3- to 5-week-old C57BL/6 female mice (Charles River or Daehan BioLink). Animals were maintained with food and water *ad libitum* under a 14-h light/10-h dark cycle. The guidelines of the Institutional Animal Care and Use Committees were followed for all animal procedures.

Oocyte collection

GV oocytes were recovered from the ovaries of mice that had been administered 5IU of a pregnant mare's serum gonadotrophin (PMSG, Calbiochem). Oocytes were released from the ovaries by puncturing with a fine needle and were placed in M2 medium (Millipore) supplemented with 200 μ M of IBMX (Sigma) to prevent GVBD. Only oocytes with an intact layer of cumulus cells were recovered, and cumulus cells were subsequently removed by repeated pipetting with a mouth-operated micropipette.

To obtain MI oocytes, the GV oocytes were washed extensively in IBMX-free M16 medium and cultured at 37°C in 5% CO₂ for 8 h.

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MI I oocytes were obtained from the superovulated mice with 5IU of PMSG followed by 5IU of human chorionic gonadotrophin (hCG, Sigma) 48 h apart. Ovulated oocytes were released from the ampullae of the oviducts at 16 h post-hCG. The cumulus cells were removed by a brief exposure to 0.1 mg/ml of hyaluronidase (Sigma) in M2 medium. Parthenogenetic activation of MII oocytes was achieved by washing the oocytes in Ca^{2+} -free media containing 10 mM SrCl_2 (Sigma).

Microinjection

Oocytes were microinjected as described previously (Oh et al., 2010). Briefly, antibodies (200 ng/ μl) or cRNAs (500 ng/ μl) were microinjected into the cytoplasm of MII oocytes in M2 medium. Approximately 10 μl of solution containing antibodies or cRNAs was injected per oocyte. Antibodies for microinjection were purchased from Santa Cruz (Cdc25B, sc-326; Cdc25C, sc-327). Following microinjection, oocytes were cultured in M16 medium (Millipore) at 37°C in 5% CO_2 atmosphere and a pronuclear formation was observed using an inverted microscope (DMI 4000B; Leica).

Preparation of Cdc25B cRNAs

The full-length cDNA encoding the mouse Cdc25B were purchased from Origene. The catalytically inactive mutants of Cdc25B (C483S) were generated using site-directed mutagenesis. cRNAs for microinjection were made *in vitro* using the mMESSAGE mMACHINE kit (Ambion). After *in vitro* transcription, cRNAs were immediately polyadenylated using the Poly (A) Tailing Kit (Ambion) and purified with the RNeasy Mini kit (Qiagen). The concentration of cRNAs was determined by OD260 and agarose gel electrophoresis.

RT-PCR

Total RNAs were extracted from GV, MI or MII oocytes using the RNeasy Plus Mini Kit (Qiagen) followed by reverse transcription (RT) using Sensiscript RT kit (Qiagen). PCR was performed using the following primers: for Cdc25B, GATGGAA-GTAGAGGAGC and CTTCCAGGGGTGTACAC; for GAPDH, ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA. PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 sec, extension at 72°C for 30 s, and final extension at 72°C for 10 min.

Western blotting and immunostaining

For Western blotting, oocytes were collected in phosphate buffered saline (PBS) containing 1% polyvinylpyrrolidone (PVP) and frozen in SDS sample buffer. Western blotting was performed using antibodies against Cdc25B (Santa Cruz), cyclin B (Abcam) or α -tubulin (Abcam). For immunostaining, oocytes were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS. Oocytes were incubated with DAPI and FITC-conjugated Lens culinaris agglutinin (LCA) for DNA and cortical granule staining, respectively. Immunostaining was visualized using an inverted confocal microscope (TCS SP5; Leica).

Phosphatase activity assay

The activity of Cdc25B phosphatase was determined by the modified H1 kinase assay. Briefly, Cdc25B proteins were immunoprecipitated from GV or MII oocytes. GV oocytes were lysed in 5 μl of lysis buffer (10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM p-nitrophenyl phosphate, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, and 5 mM EGTA) and preincu-

bated with Cdc25B immunoprecipitates for 10 min. The kinase reaction was initiated by the addition of 5 μl of kinase buffer [24 mM p-nitrophenyl phosphate, 90mM β -glycerophosphate, 24 mM MgCl_2 , 24 mM EGTA, 0.2 mM EDTA, 4.6 mM sodium orthovanadate, 4 mM NaF, 1.6 mM dithiothreitol (DTT), 60 $\mu\text{g}/\text{ml}$ aprotinin, 60 $\mu\text{g}/\text{ml}$ leupeptin, 2.2 μM cAMP-dependent protein kinase inhibitor (PKI), 0.6 mM ATP, 2 mg/ml histone (type III-S; Sigma) with 500 $\mu\text{Ci}/\text{ml}$ γ - ^{32}P ATP (3,000 Ci/mmol; Amersham)]. The reaction was conducted for 10 min at 30°C and terminated by the addition of 10 μl of 2XSDS sample buffer and boiling for 3 min. Following SDS-PAGE, the gel was transferred to a PVDF membrane and analyzed by autoradiography.

Statistical analysis

Data are representative of at least three independent experiments unless otherwise specified. Values were analyzed by one-way ANOVA or Student's *t*-test, and $p < 0.05$ was considered statistically significant.

RESULTS

To investigate the role of Cdc25B during MII arrest, we first examined the expression of Cdc25B at different stages of meiotic maturation. RT-PCR analysis showed that Cdc25B mRNA expressed continuously during the meiotic cell cycle (Fig. 1A). Consistent with this, immunoblotting analysis showed that Cdc25B protein was expressed from the germinal vesicle (GV) to MII stages with a dramatic mobility shift (Fig. 1B), suggesting the post translational modifications during meiotic maturation. It has been known that Cdc25B undergoes a major mobility shift with an extensive phosphorylation during mitotic progression (Gabrielli et al., 1997). Thus, we investigated whether Cdc25B is phosphorylated during the meiotic cell cycle in mouse oocytes. The mobility shift of Cdc25B during MII arrest was significantly decreased by the treatment of a protein phosphatase (Fig. 1C), indicating that the increase in molecular weight of Cdc25B is due to the hyperphosphorylation. Next, to investigate the physiological meaning of the hyperphosphorylation of Cdc25B, we measured the phosphatase activity of Cdc25B at the GV and MII stages. Since Cdc25B dephosphorylates and hence activates Cdk1, we measured the activity of Cdk1 using histone H1 as the target substrate (Hasepass and Hoffmann, 2004; Tumurbaatar et al., 2011). Interestingly, the Cdk1 activity was significantly increased when incubated with Cdc25B of MII oocyte lysates (Fig. 1D), implying that Cdc25B phosphorylation during MII arrest is associated with an activation of Cdc25B. Consistent with our data, it has been reported that the hyperphosphorylation of Cdc25B is correlated with increased phosphatase activity in mitosis (Gabrielli et al., 1997).

To examine the role of Cdc25B during MII arrest, specific neutralizing antibodies were injected into MII oocytes. Because Cdc25C is dispensable for the meiotic cell cycle (Chen et al., 2001), antibody against Cdc25C was used as a negative control with normal IgG. Surprisingly, chromatins were dencondensed and subsequently a pronucleus-like structure was formed when the oocytes were injected with Cdc25B antibody (Fig. 2A). Considering that the pronuclear structure is indicative of the progression of cell cycle from metaphase to interphase, this result indicates that oocytes were released from MII arrest. In contrast, little effect was shown in the oocytes injected with either normal IgG or Cdc25C antibody (Fig. 2A). Moreover, the formation of the pronucleus-like structure occurred without cortical granule exocytosis (Fig. 2B), one of the earliest Ca^{2+} -mediated events after egg activation (Tahara et al., 1996), ex-

Fig. 1. Expression and phosphorylation of Cdc25B during meiotic cell cycle. (A, B) RT-PCR (A) and Western blot analysis (B) of Cdc25B phosphatase during meiotic cell cycle in mouse oocytes. Samples were collected after the oocytes had been cultured for 0 and 8 h at the GV and MI stages, respectively. MII oocytes were obtained from the oviducts 16 h after hCG injection. Each lane contains 100 oocytes. (C) Phosphorylation of Cdc25B during meiotic cell cycle. MII oocyte lysates were treated with λ-phosphatase (PPase) and subjected to SDS-PAGE followed by immunoblotting. GV and MII oocytes were used as controls for the mobility shift. (D) Phosphatase activity of Cdc25B. Immunoprecipitates of Cdc25B (IP-Cdc25B) from GV or MII oocytes were incubated with

Histone H1 and GV oocyte lysates in the presence of [γ-32P]ATP for 10 min, and incorporation of 32P was detected by autoradiography. The amounts of loaded protein were determined by immunoblot. The arrowhead indicates the heavy chains of IgG.

Fig. 2. Partial release from MII arrest by disruption of Cdc25B function. (A) Effect of Cdc25B antibody injection on pronuclear formation (PN). MII oocytes were injected with antibodies against Cdc25B, and oocytes with pronuclei were counted after 8 hours. Normal IgG and Cdc25C antibody were used as negative control. Data include the mean ± SEM from at least three independent experiments. *p < 0.005. (B) MII oocytes injected with Cdc25B antibody were stained for DNA and cortical granule (CG). Activated eggs were used as a control for CG exocytosis, one of the earliest Ca²⁺-induced events after egg activation. Normal IgG and Cdc25C antibody were used as negative controls. Note that the pronucleus-like structure was formed in the absence of CG exocytosis in the oocytes injected with Cdc25B antibody. The arrowheads indicate the pronuclei with decondensed chromosomes. The scale bar is 20 μm.

cluding the possibility of artificial Ca²⁺ oscillation during microinjection. This is consistent with our previous finding that the disruption of Cdc25A activity during MII arrest led to the release from MII arrest in mouse oocytes (Oh et al., 2013). Thus, our results suggest that the cooperative function of Cdc25 phosphatases is required to maintain MII arrest in mouse oocytes.

Since Cdc25B is required to maintain MII arrest, we investigated whether Cdc25B is inactivated during the release from MII arrest. Because the hyperphosphorylation of Cdc25B represents the increased activity of phosphatase, we examined the mobility shift of Cdc25B during the onset of anaphase. Interestingly, the low molecular weight form of Cdc25B, which represents the decreased phosphatase activity, appeared immediately after the activation of oocytes and this occurred prior to cyclin B degradation (Figs. 3A and 3B). This result suggests that Cdc25B is inactivated during the onset of anaphase, but also implies that Cdk1 is immediately inactivated, probably independent of cyclin B degradation.

In the reverse experiment, sustained activity of Cdk1 driven by overexpression of Cdc25B significantly disturbed MII exit, whereas oocytes expressing catalytically inactive (dead) Cdc25B

were normally activated with calcium stimulation (Fig. 3C). This result suggests that failure to disrupt the Cdk1 activity can maintain the balance in favor of the metaphase state. Indeed, it has been shown that oocytes with constitutively active Cdk1 are resistant to activation in mouse oocytes (Oh et al., 2011; 2013).

DISCUSSION

In this study, we have shown that Cdc25B participates in maintaining MII arrest in mouse oocytes by regulating MPF activity. Although a regulation of MPF activity by Cdc25B-mediated dephosphorylation of Cdk1 is well established, a prevailing concept in the cell cycle is that the metaphase to anaphase transition is triggered by the proteolytic degradation of cyclin B through the activation of APC/C. However, accumulating evidence shows that the degradation of cyclin B may not be the only mechanism to inactivate Cdk1 during the exit from metaphase (Chesnel et al., 2006; Chow et al., 2011; D'Angiolella et al., 2007; Nishiyama et al., 2000). In mouse oocytes, the inhibitory phosphorylation of Cdk1 at Tyr15 immediately appears during MII exit and this phosphorylation is mediated by the

Fig. 3. Inactivation of Cdc25B during the release from MII arrest. (A) Western blot analysis of Cdc25B during the exit from MII arrest. MII oocytes activated with strontium were prepared at different time and subjected to SDS-PAGE followed by immunoblotting for Cdc25B or cyclin B. (B) Relative intensities of non-phosphorylated Cdc25B and cyclin B during egg activation were assessed using Image J software. Note that a significant amount of low molecular weight form of Cdc25B appeared 15 min after activation when no obvious difference was observed in the cyclin B level. (C) Overexpression of Cdc25B phosphatase. MII oocytes injected with Cdc25B wild-type (WT) or catalytically inactive (dead) mRNAs were activated with strontium and oocytes with a pronuclei-like structure were scored after 8 hours. Data include the mean $\pm$ SEM from three independent experiments. * $p < 0.05$.

reactivation of Wee1B kinase (Oh et al., 2011). In addition, Swe1, yeast homologue of Wee1, is hyperphosphorylated and thereby inactivated during mitosis and this phosphorylation transiently disappears during the exit from mitosis, just prior to cyclin B degradation (Harvey and Kellogg, 2003). Consistent with these, our data show that Cdc25B dephosphorylation and inactivation occur prior to the cyclin B degradation during the exit from meiosis in mouse oocytes.

Since Cdc25A and B seem to be involved in maintaining MII arrest in mouse oocytes (Oh et al., 2013), it is likely that both phosphatases cooperatively regulate Cdk1 activity, although Cdc25A activity seems to be more critical. It could be possible that Cdc25A and B dephosphorylate Cdk1 in different subcellular compartments of the MII oocytes. For instance, Cdc25A dephosphorylates Cdk1 that resides in the spindle, whereas Cdc25B functions in the cytoplasm. Indeed, it has been shown that Cdc25A and B localize in the nucleus and cytoplasm, respectively, during GV arrest (Solc et al., 2008). Nevertheless, we cannot exclude the possibility that additional properties of Cdc25A and B are present for a cooperative regulation of Cdk1 activity during MII arrest in mouse oocytes.

Collectively, our data reveal interesting possibilities for MPF regulatory mechanisms in mouse oocytes. During MII arrest, MPF activity is maintained by the Cdc25-mediated positive feedback loop. The disruption of this loop by Cdc25 inactivation could reduce Cdk1 activity to levels that are competent to stimulate APC/C. This would subsequently lead to cyclin B degradation and a concomitant drop in kinase activity. Consistent with this, it has been shown that in frog oocytes Cdc25C is inactivated during mitotic exit (Forester et al., 2007; Hutchins et al., 2003). It is also interesting to note that the inhibitory phosphorylation of Cdk1 rapidly appears during MII exit and this would be due to the reactivation of Wee1 kinases that counteract Cdc25 function (D'Angioliella et al., 2007; Oh et al., 2011). Therefore, it is most likely that the activity of MPF during MII arrest is maintained by the balance between Cdk1 inhibitory kinases, Wee1, and the counteracting phosphatases, Cdc25. When oocytes exit from MII arrest, Cdc25 is inactivated and

Wee1 is reactivated, and Cdk1 kinase activity is thereby rapidly and transiently decreased. This initial decrease of Cdk1 activity is further promoted by the proteolytic degradation of cyclin B, which ensures irreversible progression of the cell cycle to interphase. Thus, the concerted effort of phosphorylation and dephosphorylation of Cdk1 and the synthesis and degradation of cyclin B plays a role in fine-tuning the activity of Cdk1 during MII arrest and exit in mouse oocytes.

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