CDC6 Requirement for Spindle Formation During Maturation of Mouse Oocytes

Martin Anger, Paula Stein, and Richard M. Schultz

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018

ABSTRACT

A master regulator of DNA replication, CDC6 also functions in the DNA-replication checkpoint by preventing DNA rereplication. Cyclin-dependent kinases (CDKs) regulate the amount and localization of CDC6 throughout the cell cycle; CDC6 phosphorylation after DNA replication initiation leads to its proteolysis in yeast or translocation to the cytoplasm in mammals. Overexpression of CDC6 during the late S phase prevents entry into the M phase by activating CHEK1 kinase that inactivates CDK1/cyclin B, which is essential for the G2/M-phase transition. We analyzed the role of CDC6 during resumption of meiosis in mouse oocytes, which are arrested in the first meiotic prophase with low CDK1/cyclin B activity; this is similar to somatic cells at the G2/M-phase border. Overexpression of CDC6 in mouse oocytes does not prevent resumption of meiosis. The RNA interference-mediated knockdown of CDC6, however, reveals a new and unexpected function for CDC6; namely, it is essential for spindle formation in mouse oocytes.

INTRODUCTION

During the relatively long period of oocyte growth and maturation, unscheduled DNA replication is prevented by repressing CDC6 (cell division cycle-6 homologue) function [1]. CDC6 plays an essential role in DNA replication, because it is required for loading prereplication complexes (pre-RCs) during licensing of DNA for replication [2]. Assembly of pre-RCs on DNA during late mitosis and the early G1 phase is initiated by loading origin-recognition complex (ORC) proteins followed by CDC6 and Cdt1, which are essential for recruiting minichromosome maintenance (MCM) proteins into the complex [3–5]. Once DNA replication is initiated, CDC6 is no longer required and is removed from pre-RCs. This step involves cyclin-dependent kinases (CDKs) phosphorylating consensus sites located in the amino terminal portion of CDC6 and results in changing its stability or localization. The nuclear concentration of CDC6 is highest during the G1 phase, when CDK activity is low, whereas during the G2 and early M phases, when CDK activity is high, CDC6 is inactivated by proteolysis in yeast or by translocation into the cytoplasm in mammals [6, 7]. This suggests that different strategies regulate the activity of pre-RCs in yeast and mammals [8–11].

Overexpression of CDC6 during the late S phase activates CHEK1 kinase. This prevents cyclin B/CDK1 activation and leads to cell-cycle arrest at the G2/M-phase transition [12]. Detailed analysis of this phenomenon in Xenopus laevis has demonstrated that CDC6 activates CHEK1 directly and that MCM proteins as well as binding of CDC6 to chromatin are not required [13].

The difference in how the amount of CDC6 protein is regulated after the initiation of DNA replication in different eukaryotes raises the possibility that CDC6 has other functions during the late S phase and mitosis in mammals. To investigate this possibility in mammalian somatic cells, however, is difficult, both because a CDC6-specific inhibitor is lacking and because a Cdc6 knockout or an RNA interference (RNAi)-mediated knockdown would be lethal. Mouse oocytes are a potentially suitable system to explore this possibility. Mouse oocytes are arrested in the first meiotic prophase after DNA replication; this is similar to being arrested at the G2/M-phase transition. Cyclin B/CDK1 (i.e., M-phase promoting factor [MPF]) activity is low in these oocytes, which is similar to somatic cells at the G2/M-phase transition. Resumption of meiosis entails an abrupt activation of MPF that leads to dissolution of the nuclear membrane (i.e., germinal vesicle breakdown [GVBD]), two rounds of chromosome segregation with no intervening DNA replication, and arrest at metaphase II [14].

We first analyzed whether overexpression of CDC6 inhibits GVBD. We found that in contrast to somatic cells, oocytes tolerate high levels of CDC6; that is, the oocytes mature normally and arrest at metaphase II. Reducing CDC6 by RNAi demonstrated that although GVBD occurred and chromosomes condensed, a spindle failed to form, suggesting a new function for CDC6 that is not related to DNA replication.

MATERIALS AND METHODS

Oocyte Collection, Culture, and Microinjection

Fully grown, germinal vesicle (GV)-intact oocytes were obtained from eCG-primed, 6-wk-old, female CF-1 mice (Harlan) and freed of attached cumulus cells as previously described [15]. The collection medium was bicarbonate-free minimal essential medium (Earle salt) supplemented with 3 mg/ml of polyvinylpyrrolidone (PVP) and 25 mM Hepes (pH 7.3). Germinal vesicle breakdown was inhibited by adding 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX) to the isolation or culture media. Oocytes were cultured in CZB medium [16] containing 0.2 mM IBMX and cultured in an atmosphere of 5% CO2 in air at 37°C. Oocytes were microinjected in bicarbonate-free Whitten medium [17] supplemented with 10 mM Hepes (pH 7.3) and 0.2 mM IBMX with 5 pl of the RNA solution; the injections were performed as previously described [18]. Typically, greater than 70% of oocytes injected with either Egfp or Cdc6 double-stranded (ds) RNA survived, and virtually all the surviving oocytes underwent GVBD. The dsRNA was diluted to 1–2 μg/μl and mRNAs to 0.5 μg/μl for injections. After microinjection, oocytes were cultured in CZB plus IBMX for 2 h when mRNA was injected and 24 h when dsRNA was injected. When required, the injected oocytes were matured by washing and culturing them in IBMX-free CZB medium for 18 h.

All animal experiments were approved by the Institutional Animal Use...
and Care Committee and were consistent with National Institutes of Health guidelines.

**RNA Isolation and Quantification**

Total RNA from 5 to 50 oocytes or eggs was isolated using the Absolutely RNA MicroPrep Kit (Stratagene, La Jolla, CA). The reverse transcription reaction, primed with random hexamers, was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Total RNA isolated from 5 to 50 oocytes was reverse transcribed in 20-μl reactions. The resulting cDNA was quantified by real-time polymerase chain reaction (PCR) using SybrGreen and detecting the threshold cycle with an ABI Prism 7000 (Applied Biosystems, Foster City, CA). Histone H1foc was used as an internal standard. The following primers were used: H1foc 5'-CTGCAAGTGCCATGAGTAAGA-3' and CDC6, 5'-TTGCTGCGATCCAGTTCTGTG-3', and CDC6, 5'-TGCTGCGATCCAGTTCTGTG-3' and 5'-CTGCAAGTGCCATGAGTAAGA-3'.

**Mutagenesis of Phosphorylation Sites**

The QuickChange Multisite-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to produce mouse CDC6 with serine residues 55, 75, and 108 mutated to alanine or aspartic acid residues. The template for in vitro transcription was a pCMV-SPORT6 vector containing a mouse full-length Cdc6 cDNA (clone 4946685, GenBank accession no. BG919477) that was purchased from Invitrogen. The mutated cDNA was completely sequenced and used as a template for in vitro transcription.

**In Vitro Synthesis of dsRNA and Capped RNA**

Two dsRNAs were used to target mouse Cdc6 mRNA. The pT7T3D-PacI vector containing a full-length mouse Cdc6 cDNA was purchased from Invitrogen (clone 477516, GenBank accession no. AI510027). The BglII/NotI fragment was excised from the plasmid to obtain a 1160-base pair fragment that was used as a template for in vitro transcription. The plasmid was linearized using XhoI and HindIII, purified by agarose gel electrophoresis, and used as the template for SP6 and T7 RNA polymerases (Promega, Madison, WI) to obtain sense and antisense RNAs. In vitro transcription and annealing were performed as described previously [19]. The second Cdc6 dsRNA was obtained using MEGAscript RNAi Kit (Ambion, Austin, TX). A PCR strategy in which the T7 sites were added on both sides of the template was used to generate template for in vitro transcription. The Egfp dsRNA also was prepared as a control dsRNA for injections. Primers for Cdc6 were 5'-AGGATCTCTAATACGACTCACTATAGGGAGAGCGCTTTACTTGTACA3' and 5'-AGGATCTCTAATACGACTCACTATAGGGAGAGCGCTTTACTTGTACA3'. The length of the dsRNA fragment was 780 bp for Cdc6 and 712 bp for Egfp.

Polyadenylated capped mRNA for injections was obtained using mMESSAGE mMACHINE T7 Kit and Poly(A) Tailing Kit (Ambion). The template for in vitro transcription was a pCMV-SPORT6 plasmid containing full-length mouse Cdc6 cDNA or point-mutated Cdc6 cDNA, each digested with BanII. The RNA was purified using RNasey Mini Kit (Qiagen, Inc., Valencia, CA).

**Antibodies**

The CDC6 was detected using monoclonal antibody sc-9964 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); a dilution of 1:100 was used for Western blot analysis and 1:50 for immunofluorescence. β-Tubulin was detected with monoclonal antibody T4024 (Sigma-Aldrich, St Louis, MO) at a 1:500 dilution for immunofluorescence. Phosphoserine 10 in histone H3 was detected with 0.5-570 antibody from Upstate Biotechnology, Inc. (Lake Placid, NY) using a working dilution of 1:500 for immunofluorescence. Lamin A was detected with rabbit polyclonal antibody 2032 (Cell Signaling Technology, Inc., Beverly, MA) at a 1:100 dilution for immunofluorescence. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were Cy5-conjugated anti-rabbit or anti-mouse, and Cy3-conjugated anti-mouse antibody. Fluorescence was detected on a Leica TCS SP laser-scanning confocal microscope.

**Immunoblotting**

Samples were separated in a 10% SDS-PAGE gel and transferred to a polyvinylindene fluoride (PVDF) membrane using semidry transfer. Membranes were blocked with 5% fetal calf serum or 5% bovine serum albumin (Sigma-Aldrich) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h at room temperature. Primary and secondary antibodies were diluted in TTBS, and the membrane was washed after each incubation. The signal was developed using ECF Western Blotting System (Amersham Biosciences, Piscataway, NY) and detected using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Kinase Assays**

The activities of mitogen-activated protein kinase (MAPK) and MPF in single eggs were determined as previously described [19].

**Statistical Analysis**

One-way ANOVA or Student t-test was used to evaluate the difference between groups, and differences at P < 0.05 were considered to be significant. Prism software (Graph Pad Software Inc., San Diego, CA) was used to perform statistical analyses.

**RESULTS**

**CD6 protein and mRNA Expression During Maturation**

Previous reports have shown that in various species, including mouse, CDC6 is not present in growing oocytes and is detected after resumption of meiosis [1, 20, 21]. We quantified the amount of Cdc6 mRNA before meiotic maturation in GV-intact oocytes and after maturation in metaphase II-arrested eggs using real-time RT-PCR. We found that the amount of Cdc6 mRNA decreases by approximately 50% following maturation (Fig. 1A). Immuno blot analysis revealed that CDC6 protein was not detected in GV-intact oocytes but was detected after GVBD (Fig. 1B). This maturation-associated increase was confirmed by immunochemistry. No detectable signal was observed in GV-intact oocytes, but a clear signal was localized to chromosomes of the metaphase II-arrested egg (Fig. 1C). Thus, degradation of Cdc6 mRNA was apparently coupled with its translation.

**CDC6 Overexpression During Resumption of Meiosis**

Overexpression of CDC6 in somatic cells blocks the cell cycle at the G2/M-phase transition by activating CHECK1 kinase, which in turn prevents activation of cyclin B/CDK1 complex that permits this transition [12]. As mentioned above, CDK1 activity is low in vertebrate oocytes arrested at the G2/M-phase transition and in somatic cells at the G2 phase. Activation of CDK1 is required for GVBD. We were
interested in establishing if the overexpression of CDC6 in oocytes inhibits GVBD by inhibiting CDK1 activation. Full-length Cdc6 mRNA without the 3′-untranslated region (UTR) regulatory sequences was prepared in vitro and microinjected into oocytes blocked at the GV stage with IBMX [18]. A control group was injected with in vitro-transcribed Egfp mRNA. Both groups of oocytes were arrested at the GV stage for additional 2 h after microinjection to allow expression and then matured for 18 h. Accumulation of the newly synthesized proteins was detected within 2 h following microinjection of the mRNAs (data not shown). Results of three independent experiments indicated no difference in the extent of resumption of meiosis following injection with either Egfp or Cdc6 mRNA (Fig. 2A), and no difference was found in the kinetics of GVBD in both groups (data not shown). Thus, overexpression of CDC6 presumably did not inhibit CDK1 activation.

Immunolocalization of CDC6 in microinjected oocytes showed that ectopically expressed CDC6 was localized on chromatin and the spindle, which was similar to that of endogenous CDC6 (Figs. 1C and 2B). The higher level of fluorescence in the oocytes injected with Cdc6 mRNA compared with that in controls was confirmed by immunoblot analysis (Fig. 2C). In summary, the results of these experiments indicate that overexpression of CDC6 by at least 30-fold (as determined by quantifying the signal intensity in Fig. 2C) when compared to the endogenous amount of CDC6 does not inhibit CDK1 activation.

Overexpression of Selected CDC6 Mutants

Previous work has shown that selected mutants of CDC6, including S74 converted to alanine and deletion of the Cy motif, are less efficient in arresting the cell cycle, whereas mutation of other serine sites (S54 and S107)
showed the same efficiency as wild-type CDC6 [12]. To analyze whether the phosphorylation state of CDC6 is important for blocking meiotic progression, we generated Cdc6 mRNAs with serines 55, 75, and 108 mutated into alanines (A mutation) or into aspartic acid (D mutation), and we injected mRNAs encoding these mutant forms into oocytes. Results of two independent experiments showed that both mutations did not increase the ability of CDC6 to prevent resumption of meiosis (Fig. 3A).

In mammals, CDC6 phosphorylation is responsible for its translocation to the cytoplasm; the unphosphorylated form is retained in the nucleus [22–24]. When we analyzed the localization of CDC6 mutants in oocytes before GVBD, we found that the A mutation was almost exclusively localized in the nucleus, which is consistent with previous results obtained in somatic cells (data not shown). Both immunolocalization (Fig. 3B) and immunoblotting (Fig. 3C) of eggs after maturation revealed a higher level of expression of the A form compared to the D form, even though similar amounts of the corresponding mRNAs were injected. The low level of expression of the D form precluded its localization in GV-intact oocytes.

**CDC6 Is Essential for Spindle Formation During Oocyte Maturation**

We used RNAi to assess the function of CDC6 in resumption of meiosis (i.e., to test whether CDC6 has additional functions beyond DNA replication). Two different dsRNAs (length, 1160 and 780 bp) were prepared to target Cdc6 mRNA in GV-intact oocytes. After 24 h in culture medium containing IBMX to inhibit maturation, the amount of Cdc6 mRNA was assayed by real-time PCR. As antici-
pated, the amount of Cdc6 mRNA was dramatically reduced (Fig. 4A). When the oocytes were allowed to resume meiosis by transferring them to inhibitor-free medium, the maturation-associated increase in CDC6 protein was not observed, as evidenced by a decrease in the intensity of the fluorescent signal (Fig. 4B).

When these injected and matured oocytes were analyzed morphologically, it became apparent that although they underwent GVBD, they failed to reach metaphase I (MI); this effect was consistently observed in several experiments using long (1160-bp) or short (780-bp) dsRNA. Morphological analysis of the injected oocytes showed that the first polar body was not extruded and that DNA formed a hollow sphere, as revealed by optical sectioning, instead of a metaphase plate (Fig. 5B). We did not detect a spindle in oocytes injected with Cdc6 dsRNA using a β-tubulin antibody; in contrast, a spindle was detected in oocytes injected with Egfp dsRNA (Fig. 5A). Although the chromosomes were condensed (a hallmark of cells undergoing mitosis/meiosis, because they stained for histone H3 phosphorylated on S10), they did not form visible bivalents (Fig. 5, C and D). In addition, the oocytes had undergone nuclear envelope breakdown, because lamin A was not detected (data not shown). Time-course studies revealed that the spindle never formed (data not shown).

In yeast and in mammals, CDC6 directly interacts with cyclin/CDK complexes, but the physiological importance of this interaction in mammals is not known [23, 25]. Activities of MPF and MAPK were measured in Cdc6 dsRNA-injected oocytes to establish that the inhibitory effect on progression to MI was not because of the failure of these kinases to become fully activated. No apparent difference was noted in the activities of these kinases following maturation of oocytes injected with either Cdc6 or Egfp dsRNA (Fig. 6).

**DISCUSSION**

We report that CDC6 protein is not detected in GV-intact oocytes and that the appearance and accumulation of CDC6 protein is coupled with a maturation-associated decrease in Cdc6 mRNA. Recent results from mouse and other species indicate that this probably is a common mechanism to prevent unwanted DNA replication throughout oocyte growth and maturation [1]. This expression pattern (i.e., a maturation-associated increase in protein but decrease in mRNA) is reminiscent of that observed for dormant maternal mRNAs that are recruited during maturation. For example, Mos and cyclin B1 mRNAs contain a cytoplasmic polyadenylation element (CPE) located in their 3'-UTR, and recruitment of the mRNA leads to its translation and degradation [26]. Consistent with this proposal is that a
CPE of the same sequence present in Mos and cyclin B1 mRNA also is present in the 3′-UTR of the mouse Cdc6 mRNA. The appearance of Cdc6 in the metaphase II-arrested egg aptly positions it to initiate DNA replication following egg activation.

As mentioned in Results, overexpression of Cdc6 in somatic cells blocks the cell cycle at the G2/M-phase transition via CHEK1 kinase [12]. Our results show that in contrast to somatic cells, resumption of meiosis (i.e., the G2/M-phase transition) is not prevented by overexpression of Cdc6. This difference could be explained by the requirements of different CDC25 isoforms for cyclin B/CDK1 activation in somatic cells and in mouse oocytes. CDC25B, which is essential for meiotic resumption in mouse oocytes, is dispensable in the somatic cell cycle [27]. In somatic cells, an activated DNA-replication checkpoint inhibits CDC25A and CDC25B, with MAPK being required for CDC25B inhibition [28, 29]. In mouse oocytes, however, MAPK is activated after MPF because of the requirement of different CDC25 isoforms for cyclin B/CDK1 activation in somatic cells and in mouse oocytes. CDC25B, which is essential for meiotic resumption in mouse oocytes, is dispensable in the somatic cell cycle [27].

We also observed that Cdc6 with N-terminal serines mutated to aspartic acid is less stable in oocytes. This is reminiscent of yeast, in which Cdc6 phosphorylation targets its destruction [31–34], and differs from mammals, in which phosphorylation promotes nuclear export of Cdc6 [22–24]. However, the amount of CDC6 is relatively constant throughout cell cycle [9, 11]. Even so, it should be noted that data regarding the stability of exogenous Cdc6 in mammalian cells are conflicting. For example, both mutations (CDK consensus serines into alanines or aspartic acid) are stably expressed from plasmids in HeLa cells [35], whereas using cell extracts, exogenous CDC6 is degraded in CDK-dependent fashion during the S phase [8].

In mammals, Cdc6 is not destroyed after the initiation of DNA replication; rather, it persists bound to chromatin throughout the cell cycle, suggesting that it may have other functions during the late S phase or mitosis [35]. In support of this hypothesis, the present results show that Cdc6 is critical for meiotic progression of mouse oocytes, because oocytes with a reduced amount of Cdc6 fail to form a spindle.

An inactive form of Cdc6 also can arrest yeast in mitosis [36]. Deleting both Cdc6 alleles in yeast results in incomplete mitosis, with randomly distributed chromosomes and cells with a DNA content less than 1C. Mutation in the Walker A motif, which is essential for ATP binding and hydrolysis [36], results in expression of a nonfunctional Cdc6, and the cells arrest in anaphase before chromosome segregation and have elongated spindles. This block, which is independent of a DNA-replication checkpoint and requires both an intact Cy box (mediating the binding of proteins to CDK cyclins in eukaryotes [36]) and CDK phosphorylation consensus sites, is overcome by increased CDK activity. Of interest is that in mouse oocytes with decreased Cdc6 protein, CDK1 activity displays its normal maturational increase in activity, yet the oocytes do not form a spindle and enter MI.

Other DNA replication proteins also are involved in chromosome condensation and distribution during mitosis. The ORC subunits 2, 5, and 6 and MCM10, which are all involved during the initial steps of DNA replication, also are required during mitosis, because decreasing the amount of these proteins leads to incorrect chromosome condensation and distribution or defects in cytokinesis [37–41]. In this regard, Cdc6 also appears to be involved in chromosome condensation, because decreasing the amount of oocyte CDC6 results in abnormal chromosome condensation and failure to form bivalents.

The present results have unmasked a new function for Cdc6 that apparently is unrelated to DNA replication—namely, that Cdc6 is critical for meiotic progression of mouse oocytes, particularly formation of a meiotic spindle. The molecular mechanism underlying this new function and why the chromosomes are found condensed on the surface of a hollow sphere are the subject of future investigations.

**REFERENCES**