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CDC25A phosphatase controls meiosis I progression in mouse oocytes

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Abstract

CDK1 is a pivotal regulator of resumption of meiosis and meiotic maturation of oocytes. CDC25A/B/C are dual-specificity phosphatases and activate cyclin-dependent kinases (CDKs). Although CDC25C is not essential for either mitotic or meiotic cell cycle regulation, CDC25B is essential for CDK1 activation during resumption of meiosis. *Cdc25a* −/− mice are embryonic lethal and therefore a role for CDC25A in meiosis is unknown. We report that activation of CDK1 results in a maturation-associated decrease in the amount of CDC25A protein, but not *Cdc25a* mRNA, such that little CDC25A is present by metaphase I. In addition, expression of exogenous CDC25A overcomes cAMP-mediated maintenance of meiotic arrest. Microinjection of *Gfp-Cdc25a* and *Gpf-Cdc25b* mRNAs constructs reveals that CDC25A is exclusively localized to the nucleus prior to nuclear envelope breakdown (NEBD). In contrast, CDC25B localizes to cytoplasm in GV-intact oocytes and translocates to the nucleus shortly before NEBD. Over-expressing GFP-CDC25A, which compensates for the normal maturation-associated decrease in CDC25A, blocks meiotic maturation at MI. This MI block is characterized by defects in chromosome congression and spindle formation and a transient reduction in both CDK1 and MAPK activities. Lastly, RNAi-mediated reduction of CDC25A results in fewer oocytes resuming meiosis and reaching MII. These data demonstrate that CDC25A behaves differently during female meiosis than during mitosis, and moreover, that CDC25A has a function in resumption of meiosis, MI spindle formation and the MI–MII transition. Thus, both CDC25A and CDC25B are critical for meiotic maturation of oocytes.

Keywords: Resumption of meiosis; Meiotic maturation; Mouse oocytes; CDC25A; CDC25B; CDK1; MAPK; Spindle formation

Introduction

Meiotic maturation, which involves resumption of meiosis of prophase I-arrested oocytes, completion of the first meiotic division and arrest at metaphase II (MII), is controlled by the activity of the CDK1-cyclin B complex (Dekel, 2005; Kishimoto, 2005; Motlik and Kubelka, 1990). CDK1, in addition to being regulated by its association with a cyclin, is also negatively regulated by phosphorylation on T14 and Y15 that are located within the ATP-binding loop. The WEE1/MYT1 protein kinases mediate this phosphorylation, whereas CDC25 phosphatases are responsible for dephosphorylation of T14 and Y15, which in turn leads to CDK1 activation (Malumbres and Barbacid, 2005).

CDK1 activity in mouse oocytes is naturally inhibited by cAMP. cAMP produced by the oocyte (and not derived from the surrounding cumulus cells that are coupled to the oocyte via gap junctions) appears essential for maintenance of meiotic arrest because oocytes, which express adenylate cyclase 3 (AC3) (Horner et al., 2003), that either lack the G(s)-linked G-protein-coupled receptor (GPR3) (Freudzon et al., 2005; Mehlmann, 2005; Mehlmann et al., 2004) or in which GPR3 is inhibited by microinjected anti-GPR3 antibodies (Mehlmann et al., 2002), resume meiosis within the follicle. A maturation-associated decrease in cAMP that precedes NEBD (Schultz et al., 1983) is

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likely mediated by phosphodiesterase 3A (PDE3A) (Masciarelli et al., 2004). PKB/AKT kinase, which is important for CDK1 activation and involved in resumption of meiosis (Kalous et al., 2006), is responsible for PDE3A activation (Han et al., 2006). cAMP-mediated inhibition of maturation is likely mediated by PKA phosphorylation of WEE1B, an oocyte-specific member of WEE1/MYT1 protein kinase family (Han et al., 2005). PKA also negatively phosphorylates *Xenopus* CDC25 (Duckworth et al., 2002) and probably mouse CDC25B (Han and Conti, 2006).

CDC25B phosphatase is essential for CDK1 activation, because oocytes obtained from *Cdc25b* −/− mice fail to activate CDK1 and resume meiosis, but do so following microinjection of *Cdc25b* mRNA (Lincoln et al., 2002). Surprisingly, CDC25C is dispensable for both mitotic and meiotic cell cycles (Chen et al., 2001). *Cdc25a* −/− mice exhibit an early embryonic lethality, which would be consistent with a critical function for mitotic cell cycle regulation (Ray et al., 2007). Little is known, however, about the role of CDC25A in oocyte maturation in vertebrates. *Cdc25a* mRNA is expressed in mouse oocytes (Wickramasinghe et al., 1995) but information regarding expression at the protein level is lacking. Microinjection of *Xenopus* Cdc25a mRNA into *Xenopus* oocytes induces resumption of meiosis more potently than microinjection of CDC25C mRNA (Okazaki et al., 1996). Microinjection of bacterially expressed human CDC25A induces resumption of meiosis of *Xenopus* oocytes but the oocytes are arrested at metaphase I-stage and do not reach metaphase II (Rime et al., 1994).

In somatic cells, CDC25A regulates both G1/S- and G2/M-associated CDK-activities (Mailand et al., 2002; Molinari et al., 2000). In late G1, S and G2-phase, CDC25A protein has a very short half-life (Mailand et al., 2002) as a consequence of CHK1-mediated phosphorylation during a normal cell cycle (Zhao et al., 2002). DNA damage or inhibiting DNA replication activates CHK1 and CHK2 kinases that then lead to rapid degradation of CDC25A, thereby preventing cell cycle progression (Falck et al., 2001; Mailand et al., 2000; Molinari et al., 2000; Zhao et al., 2002). In contrast, CDC25A is stabilized by CDK1 phosphorylation on at least two residues, S17 and S115, before entry to mitosis. This mitotic form of CDC25A is stable even after ionizing radiation, which induces double-strand DNA breaks (Mailand et al., 2002). Degradation of CDC25A at anaphase and early G1 mediates APC/C^{CDH1} activation. CDC25A contains a KEN-box that is essential for degradation mediated by the APC/C but mutations in the KEN-box mutation do not affect the stability of CDC25A protein in interphase protein (Donzelli et al., 2002).

We report here that CDC25A protein is present in fully grown meiotically competent oocytes (GV-stage). In contrast to CDC25B, which resides in the cytoplasm, CDC25A is a nuclear protein. Following NEBD, there is a maturation-associated decrease in the amount of CDC25A such that only small amounts of CDC25A are present in MI and MII eggs. Over-expressing CDC25A overcomes cAMP-mediated maintenance of meiotic arrest and RNAi-mediated CDC25A knock-down indicates a role for CDC25A in both resumption of meiosis and the MI–MII transition.

Materials and methods

Oocyte collection and culture, and RNA microinjection

Mouse ovaries were obtained from 3–4 week-old PMSG-primed (C57BL/6J X BALB/c) F1 hybrid female mice. Ovaries were transferred to bicarbonate-free minimal essential medium (Earle salt) at 37 °C and supplemented with 3 mg/ml of polyvinylalcohol (PVA) and 25 mM HEPES (pH 7.3). To inhibit resumption of meiosis, the medium contained 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX). Oocytes were cultured in M-16 medium (M7292, Sigma Aldrich) at 37.5 °C in 5% CO_2 in air.

Oocytes were microinjected with 5 pl of the RNA solution using an MIS-5000 micromanipulator (Burleigh, Exfo Life Sciences, USA) and PM 2000B4 microinjector (MicroData Instrument, USA). The microinjection medium was Whitten's medium supplemented with 10 mM HEPES (pH 7.3) and 0.1 mM IBMX. Pipets for microinjection were made using P97 Pipette Puller (Sutter Instrument Company, USA).

In vitro mRNA and dsRNA production for microinjection

pCMV-SPORT6 vector containing mouse full-length *Cdc25a* (GenBank accession no. BC046296.1, MGC:66900, IMAGE:6401489), and pYX-ASC vector containing mouse full-length *Cdc25b* (GenBank accession no BC057568.1, MGC:66900, IMAGE:6401489) were purchased from imaGenes GmbH, Germany (formally RZPD German Resource Center for Genome Research).

Cdc25a and *b* mRNAs for microinjection were produced by *in vitro* transcription using mMESSAGE mMACHINE® T3 Kit (#1348, Ambion). To generate the template for transcription, full-length mouse *Cdc25 a* and *b* cDNAs were cloned into *Spe*I site (for N-terminal GFP tags) of the pBluscript-GFP vector containing a T3-promoter and *Xenopus* globin 5′UTR, 3′UTR and Kozak sequences for high mRNA stability and efficient translation initiation, respectively. This vector was obtained from Martin Anger, University of Oxford, UK, and will be described elsewhere. For *in vitro* transcription, the vectors were linearized with Asc1. After *in vitro* transcription, mRNAs were immediately polyadenylated using the Poly(A) Tailing Kit (#AM1350, Ambion). mRNAs were purified using RNeasy Mini Kit (#74104, Qiagen). *Gfp* mRNA for control microinjection was transcribed from an empty pBluscript-GFP vector.

dsRNA was produced using MEGAscript® RNAi Kit (#AM1626, Ambion). 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. Primers for *Cdc25a* were AGGATCCTAATACGACTCACTATAGGGAGAAGCTGCTGGCGGACTGTC and ACTCGAGTAATACGACTCACTATAGGGAGACAAACAGCCCGCAACGAT, for control *Gfp*, AGGATCCTAATACGACTAACTATAGGGAGAATGGTGAGCAAGGGCGAGGA and ACTCGAGTAATACGACTCACTATAGGGAGAGCGGCCGCTTTACTTGTACA. The length of the dsRNA fragment was 653 bp for *Cdc25a* and 712 bp for *Gfp*.

Both mRNA and dsRNA, in nuclease-free water (#AM9939, Ambion), were aliquoted (5 µl) at 500 ng RNA/µl and stored at −80 °C until used for microinjection.

Total RNA isolation and mRNA quantification

Total RNA from 15 or 25 oocytes was isolated using Absolutely RNA® Microprep Kit (Stratagene) and eluted with 42 µl of elution buffer. External *Gfp* mRNA (0.04 pg *Gfp* mRNA per oocyte) was added to lysed oocytes to serve as a control for quantifying RNA recovery and normalizing the RT-PCR data to the exogenously added *Gfp* mRNA. The eluted total RNA (3 µl) was used in 10 µl reaction volume using one-step QuantiTect SYBR Green RT-PCR Kit (#204243, Qiagen). Primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, USA). The following gene-specific primers were used: *Cdc25a* primers located at 3′ end of mRNA: AGAACCCTATTGTGCCTACTG and TACTCATTGCCGAGCCTATC; *Cdc25a* primers located at 5′ end of mRNA were purchased (QuantiTect Primer Assay QT01058778, Qiagen); *Gfp* primers were TTCAAGATCCGCCACAAC and GACTGGGTGCTCAGGTAG. One-step real-time RT-qPCR was done using a Chromo4 Real-time PCR detection System (Biorad), and passive ROX reference was used for reaction volume correction. The following one-step real-time qRT-PCR protocols were used: *Cdc25a 3′end*: (1) 50 °C 30 min, (2) 95 °C 15 min, (3) 94 °C 15 s, (4) 57 °C 30 s, (5) 72 °C 30 s, (6) 75 °C 3 s, (7) plate reading, (8) go to step