Cytoplasmic polyadenylation controls cdc25B mRNA translation in rat oocytes resuming meiosis

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Abstract

Resumption of meiosis in oocytes represents the entry into M-phase of the cell cycle and is regulated by the maturation-promoting factor (MPF). Activation of MPF is catalyzed by the dual specificity phosphatase, cdc25. In mammals, cdc25 is represented by a multigene family consisting of three isoforms: A, B and C. A recent report that female mice lacking cdc25B exhibit impaired fertility suggests a role for this isoform in regulating the G2- to M-transition in mammalian oocytes. Supporting the above-mentioned observation, we demonstrate herein that microinjection of neutralizing antibodies against cdc25B interfered with the ability of rat oocytes to undergo germinal vesicle breakdown (GVB). We also show accumulation of cdc25B in GVB oocytes and a transient reduction in its amount at metaphase I of meiosis. The accumulation of cdc25B was associated with its mRNA cytoplasmatic polyadenylation and was prevented by the protein synthesis inhibitor cyclohexamide as well as by the polyadenylation inhibitor cordycepin. Immunofluorescence staining revealed translocation of cdc25B to the metaphase II spindle apparatus. Taken together, our findings provide evidence that cdc25B is involved in resumption of meiosis in rat oocytes. We further demonstrate for the first time, a periodic accumulation of cdc25B throughout meiosis that is translationally regulated and involves cdc25B mRNA polyadenylation.

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Introduction

Meiosis is a particular example of cell division occurring in germ cells. The entry of the female germ cell into meiosis takes place in the fetal ovary. The meiotic division in oocytes proceeds up to the diplotene stage of the first prophase and is arrested around birth. The oocytes arrest at a stage that corresponds to the G2-phase of the cell cycle and are characterized by diffuse chromosomes surrounded by a nuclear membrane known as germinal vesicle (GV). Reinitiation of meiosis takes place in sexually mature females and is triggered in vertebrates by the pituitary gonadotropin, leutenizing hormone (LH). Mammalian oocytes can also resume meiosis spontaneously, upon their removal from the ovarian follicle (reviewed by Tsafriri & Dekel 1994). Reinitiation of meiosis represents the transition from G2- to M-phase of the cell cycle and involves dissolution of the nuclear membrane referred to as germinal vesicle breakdown (GVB), chromosome condensation, formation of the spindle of the first metaphase (MI), and segregation of the homologous chromosomes. Oocytes complete the first meiotic division by the formation of the first polar body (PBI) that is immediately followed by

their maturation into unfertilized eggs arrested at the second metaphase of meiosis (MII). Completion of the second meiotic division is triggered at fertilization by sperm penetration (reviewed by Dekel 1996).

The key regulator of meiosis is the maturation-promoting factor (MPF) (Masui & Markert 1971), which consists the catalytic p34cdc2 serine/threonine kinase and the regulatory cyclin B (Gautier *et al.* 1988, 1990). Activation of MPF is initially dependent on the availability of cyclin B that allows the formation of the p34cd2/cyclin B complex, known as pre-MPF. This pre-MPF stays inactive under the influence of the wee-1 and myt-1 kinases that phosphorylate the threonine 14 and tyrosine 15 residues of p34cdc2 (Russell & Nurse 1986, Gould & Nurse 1989, Mueller *et al.* 1995, Booher *et al.* 1997). The dual specificity phosphatase cdc25, which removes these inhibitory phosphates, converts the inactive pre-MPF into an active MPF (Gautier *et al.* 1991, Kumagai & Dunphy 1991, Millar *et al.* 1991, Strausfeld *et al.* 1991, Sebastian *et al.* 1993).

Homologs of cdc25 have been identified in different organisms including yeasts (Russell & Nurse 1986, Russell *et al.* 1989), *Caenorhabditis elegans* (Ashcroft *et al.* 1998), *Drosophila* (Edgar & O'Farrell 1989,

Alphey *et al.* 1992, Courtot *et al.* 1992), and *Xenopus* (Izumi *et al.* 1992, Kumagai & Dunphy 1992). Mitosis in fission yeast is regulated by the rate of accumulation of cdc25 during G2 (Russell & Nurse 1986). On the other hand, the amount of cdc25 in *Xenopus* oocytes is constant and its activity is regulated posttranslationally by multiple phosphorylations. Phosphorylation of cdc25 is catalyzed by members of the polo-like family of kinases (Kumagai & Dunphy 1996, Qian *et al.* 1998) as well as by p34cdc2 kinase. The reciprocal phosphorylation of cdc25 and MPF creates an autocatalytic loop that sustains the activities of these two enzymes to drive the cell cycle into M-phase (Izumi *et al.* 1992, Kumagai & Dunphy 1992, Hoffmann *et al.* 1993). Conversely, phosphatase 2A is proposed to be involved in a negative regulation of cdc25 activity (Kumagai & Dunphy 1992, Clarke *et al.* 1993, Hoffmann *et al.* 1993).

Another mechanism that participates in regulation of cdc25 action involves alteration of its cellular distribution. Localization of cdc25 is determined by the 14-3-3 protein (Conklin *et al.* 1995, Furnari *et al.* 1997, Peng *et al.* 1997, Dalal *et al.* 1999, Kumagai & Dunphy 1999) that binds the phosphatase and prevents its transport into the nucleus (Zeng & Piwnica-Worms 1999, Davezac *et al.* 2000).

In mammalian cells, cdc25 represents a multigene family consisting of A, B, and C isoforms (Galaktionov & Beach 1991, Nagata *et al.* 1991, Jinno *et al.* 1994). Each member of the mammalian cdc25 family has a role at a particular phase of the cell cycle that is determined by its substrate specificity (Sebastian *et al.* 1993). Accordingly, cdc25A regulates the G1- to S-phase transition, by dephosphorylating the Cdk2-cyclin E kinase (Hoffmann *et al.* 1994, Jinno *et al.* 1994), whereas the entry into mitosis is regulated by cdc25C (Sadhu *et al.* 1990, Millar *et al.* 1991), which elicits its action towards the p34cdc2/cyclin B complex (Gautier *et al.* 1991). Evidence regarding the role of cdc25B is still controversial. One report proposed that this form of cdc25 is required for progression into S-phase (Garner-Hamrick & Fisher 1998), while other studies have shown that cdc25B has a role in the G2-phase as a regulator of centrosomal microtubule nucleation (Millar *et al.* 1991, Gabrielli *et al.* 1996) and a starter of mitosis (Nishijima *et al.* 1997). This controversy can be possibly explained by the discovery of at least three splice variants of cdc25B that display differences in their biochemical properties (Baldin *et al.* 1997*b*).

A large body of information on the role of cdc25 in meiosis has been generated in *Xenopus* oocytes with hardly any available data on this key phosphatase in mammals. Furthermore, the specific member of the mammalian cdc25 family that functions in meiosis of mammalian oocytes is largely unknown. Early studies detected mRNA of cdc25B in murine ovaries particularly in oocytes, while mRNAs of cdc25A and cdc25C were observed in spermatids (Wickramasinghe *et al.* 1995,

Wu & Wolgemuth 1995). Conflicting results were reported by a later study that identified cdc25C mRNA in mouse oocytes further demonstrating an increase in its protein product during oocyte maturation (Mitra & Schultz 1996). Along this line, it has been shown that cdc25C is essential for progression of porcine oocytes through late diakinesis and that *de novo* synthesis of this isoform is required for the transition from meiosis in oocytes to mitosis in early embryos (Dai *et al.* 2000). However, normal fertility exhibited by cdc25C deficient female mice (Chen *et al.* 2001) suggests that this phosphatase is either irrelevant or that another protein compensates for its function in the control of meiosis. A more recent report indeed demonstrated that oocytes of mice lacking cdc25B fail to activate MPF and remain meiotically arrested (Lincoln *et al.* 2002). Taking this information into account, we examined the pattern of cdc25B expression and investigated the mode of its regulation in rat oocytes resuming meiosis.

Supporting the above-mentioned observations, we show that microinjection of neutralizing antibodies against cdc25B, but not cdc25C impaired the ability of oocytes to resume meiosis. Along this line, we demonstrate changes in cdc25B localization that are associated with the progress of meiosis. Our findings also reveal that cdc25B exhibits periodic accumulation throughout meiosis that is translationally regulated and corresponds to the oscillatory pattern of MPF activation. We show herein that cdc25B translation is mediated by polyadenylation of its mRNA.

Materials and Methods

Animals

Eighteen (weighing around 30 g) or 25-day-old (weighing around 60 g) female Wistar rats were purchased from Harlan Laboratories (Rehovot, Israel). Animals were housed under constant conditions of temperature and humidity, with lights on between 0600 and 2000 h, and food and water available *ad libitum*.

The investigation was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy of Science; Bethesda, MD, USA).

Antibodies and reagents

Cyclohexamid (CHX), isobutylmethylxanthine (IBMX), leupeptin, cordycepin and dibutyryl-cAMP (dbcAMP) were purchased from Sigma. MG132 (Z-leu-leu-CHO) was purchased from Calbiochem (Schwalbach, Germany). Syto13 was purchased from Molecular Probes (Paisley, Strathclyde UK). Goat anti-mouse-HRP, goat anti-rabbit-HRP and goat anti-mouse-Cy3 were purchased from Jackson Laboratories (West Grove, PA, USA). Monoclonal mouse anti-cdc25B antibodies were

purchased from Transduction Laboratories (San Jose CA, USA). The cdc25B peptide was purchased from Transduction Laboratories; this peptide includes the amino acids in the 109–122 position of cdc25B protein sequence. Polyclonal rabbit anti-p34cdc2 antibodies were purchased from Santa Cruz (Santa Cruz CA, USA). Monoclonal mouse anti-tubulin antibody was purchased from Sigma.

Oocytes recovery and culture

Rat oocytes that resume meiosis spontaneously upon their removal from the ovarian follicles were employed for our study. The ability to resume meiosis is acquired progressively during oocyte growth. Accordingly, oocytes that are incompetent and competent to resume meiosis are referred to as growing and fully grown, respectively. A previous study in our laboratory (Goren & Dekel 1994) demonstrated that ovaries of 18-day-old Wistar rats contain growing oocytes, while fully grown oocytes can be recovered from ovaries of 25-day-old rats, primed (48 h earlier) with 10 IU of pregnant mare's serum gonadotropin (PMSG, Chrono-gest Intervest). These two groups of animals served, therefore, as the source for the oocytes used for this study. The rats (Harlan, Rehovot, Israel) were sacrificed and their ovaries isolated and transferred into Leibovitz L-15 tissue culture medium (Gibco-BRL) containing 5% fetal bovine serum (Sigma). Follicles were punctured and the cumulus–oocyte complexes removed and placed into acidic L-15 medium (pH 6.0) to obtain cumulus-free oocytes. The oocytes were analyzed either immediately after their isolation or after the indicated times of incubation in a 37 °C humidified incubator. Growing oocytes did not resume meiosis in vitro, whereas fully grown oocytes were maintained meiotically arrested by their incubation with dbcAMP (2 mM) and the cAMP phosphodiesterase inhibitor IBMX (0.02 mM). Meiotic arrest was identified by the presence of GV. Resumption of meiosis, which was indicated by GVB, occurred spontaneously in oocytes incubated in inhibitor-free medium for 4 h. These oocytes reached MI at 8 h after isolation, with PBI being emitted at 10 h of incubation. Oocytes examined after an overnight incubation were arrested at MII.

Western blot analysis

At the end of the indicated incubation time, the oocytes were lysed in Laemmli buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) and the samples were boiled for 5 min. The proteins were electrophoretically separated on a 10% acrylamide gel, followed by their transfer to a nitrocellulose membrane. After blocking with 5% skimmed milk, the membranes were incubated with the relevant primary antibodies overnight at 4 °C

followed by the secondary antibodies for 1 h in room temperature. To confirm the specificity of the signal, the first antibody was preincubated with its relevant peptide (1:100) for 2 h at room temperature, prior to immunoblotting. The immunoreactive bands were detected by ECL (Amersham). The intensity of the signal was quantitated by computerized densitometry (quantity one).

Immunocytochemistry

Oocytes were fixed by 3% paraformaldehyde for 20 min, followed by their permeabilization with 1% triton (Sigma) for 4 min. These oocytes were incubated with monoclonal anti-cdc25B (1:200 dilution) at 4 °C, followed by further incubation with the secondary Cy3-conjugated anti-mouse antibody (1:300 dilution) for 1 h at room temperature. For fluorescent staining of the DNA, syto-13 was added along with the secondary antibody (1:1000). To confirm their specificity, the anti-cdc25B antibodies were preincubated with the relevant peptide (1:100) for 2 h at room temperature. All washes were done in GB-PBS (PBS containing 10 mg/ml of BSA and 10 mM glycine, pH 7.4). The oocytes were visualized by a laser scanning confocal system (BioRad) attached to an inverted microscope ECLIPSE TE-300 (Nikon; Melville, NY, USA).

Polyadenylation

The polyadenylation test (PAT) was preformed as described previously (Salles *et al.* 1999). Briefly, total RNA from 20 oocytes was extracted and dissolved in 5 µl double distilled water (DDW). Upon the addition of 1 µl of oligo(dt)-anchor (200 ng/µl, 5′-GCGAGCTCCGCGGCCGCGT$_{12}$–3′), the test tubes were warmed for 5 min at 65 °C, and immediately transferred to 42 °C for 2 min. This was followed by the addition of 14 µl of the reverse transcriptase (RT) cocktail [4 µl of 5×superscript RNase H⁻ RT buffer Gibco BRL); 2 µl of 0.1 M DTT; 1 µl of 10 mM dNTPs; 1 µl of RNasin (Promega), 4 µl of H$_2$O, 2 µl (200 U/µl) superscript RNase H⁻ RT (Gibco-BRL)]. The samples were incubated at 42°C for 1 h and kept at 65 °C for 15 min for RT inactivation. For the PCR, 1.2 µl of cDNAs was added to a 50 µl of standard PCR cocktail containing 0.5 µl [α-P^{32}] dATP and 25 pmol of each cdc25B mRNA specific primer (5′-CTTTTCCTTTAAGACCCCCAG-3′) and the oligo(dt)-anchor primer (amplification conditions: 93°C for 5 min; 30 cycles at 93°C for 30 s; 57°C for 1 min; 72°C for 1 min; with a final extension of 7 min at 72°C). After amplification, PCR products were ethanol-precipitated with 2.5 M ammonium acetate to remove unincorporated radioactivity. To confirm the specificity of the amplification, samples were digested by the restriction enzyme, BtsI (Promega). The radioactive products were

electrophoretically separated on 5% non-denaturing polyacrylamide gel in 0.5X TBE buffer. Gels were dried and radioactivity was determined by their exposure to X-ray film over night.

Microinjection

For microinjection, the oocytes were placed in a 50 μl drop of L-15 tissue culture medium containing dbcAMP (2 mM) and IBMX (0.02 mM), under Paraffin oil. Oocyte were microinjected by 5–7 pl of anti-cdc25B or anti-cdc25C antibodies (83 μg/ml in 75% PBS). After microinjection, the oocytes were transferred into inhibitor-free medium, further incubated for 8 h at 37 °C and monitored for GVB by differential interference contrast (DIC) microscopy (Zeiss, Germany). Oocytes microinjected with anti-IgG (83 μg/ml in 75% PBS) served as control.

Statistical analysis

The data presented represent an average of the indicated number of individual experiments and are expressed as means±s.e.m. Values were compared by F-test and considered statistically different when $P < 0.02$.

Results

Anti-cdc25B, but not anti-cdc25C inhibits GVB

As mentioned previously, the fertility of mice lacking cdc25C was not impaired. Moreover, the phosphorylation/dephosphorylation status of p34cdc2 in oocytes of these mutant mice throughout meiosis was not different from that in oocytes of wild type mice (Chen *et al.* 2001). The dispensability of cdc25C is also suggested herein by the failure of neutralizing antibodies of this isoform to interfere with meiosis upon their microinjection into rat oocytes (Fig. 1). On the other hand, microinjection of cdc25B antibodies did inhibit GVB that is the first morphological marker of reinitiation of meiosis. As expected, a large fraction (77.1±8.55%) of control, non-injected oocytes resumed meiosis spontaneously. A slightly but not significantly ($P < 0.05$) lower fraction of anti-IgG-injected oocytes lost their GV throughout 8 h of incubation (67.95±11.24%). On the other hand, microinjection of anti-cdc25B antibodies significantly ($P < 0.02$) affected the ability of the oocytes to reinitiate meiosis (GVB=44.96±8.65%).

Periodic accumulation of cdc25B during meiosis

Possible modulation of cdc25B activity throughout meiosis was studied at the translational and posttranslational levels. Western blot analysis revealed that cdc25B is expressed by growing, meiotically incompetent oocytes, and that its abundance did not change with oocyte growth (Fig. 2a–b). On the other hand, in fully
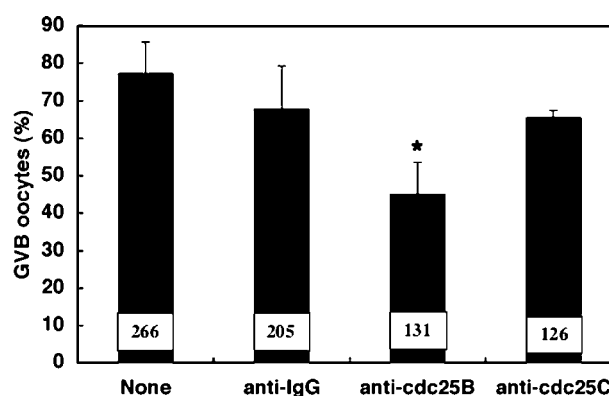

Figure 1 Reinitiation of meiosis in oocytes microinjected with anti-cdc25 antibodies. Oocytes incubated in medium containing dbcAMP in order to maintain them meiotically arrested, were microinjected with either anti-cdc25B or anti-cdc25C antibodies. After microinjection the oocytes were transferred into dbcAMP-free medium for 8 h and monitored for GVB. As a control anti-IgG antibodies were injected. Non-injected control oocytes that resume meiosis spontaneously were also included. The means±s.e.m. of GVB oocytes obtained in three experiments were calculated. Statistically significant ($P < 0.02$) differences are marked by (*). Statistical analysis was done using EXCEL. The number of oocytes examined for each point is indicated at the bottom of each column.

grown oocytes, that reinitiate meiosis a unique oscillatory pattern of cdc25B expression is demonstrated as follows. The amount of cdc25B that is elevated at GVB, decreased substantially at MI, followed by a later increase of the enzyme at MII (Fig. 2a–b). At all the developmental stages examined, the oocytes expressed one single protein that was recognized by the anti-cdc25B antibodies. Incubation of the antibodies with the relevant peptide eliminated immunoreactivity towards this protein indicating specificity towards cdc25B (Fig. 2c). Taken together, these results suggest that cdc25B action throughout meiosis is regulated by modulation of its availability rather than phosphorylation of this enzyme.

The fact that cdc25B was almost absent at MI suggested that this enzyme may be degraded in oocytes at this stage of meiosis. Examining this possibility, we found that incubation of oocytes with the specific proteasome inhibitor, MG132 (10 μM), but not the lysosome inhibitor, leupeptin (5 μM), blocked the disappearance of cdc25B at MI (Fig. 3a). These concentrations of the inhibitors have been shown previously to elicit maximal effect in our oocyte system (Josefsberg *et al.* 2000). These findings indicate that cdc25B is degraded at MI by the proteasome, but not by the lysosome. Using GAP in the Analysis package Wisconsin sequence GCG software we indeed identified the presence of KEN, a domain that is required for proteasome catalyzed proteolysis (Pfleger & Kirschner 2000), in the molecule of cdc25B.
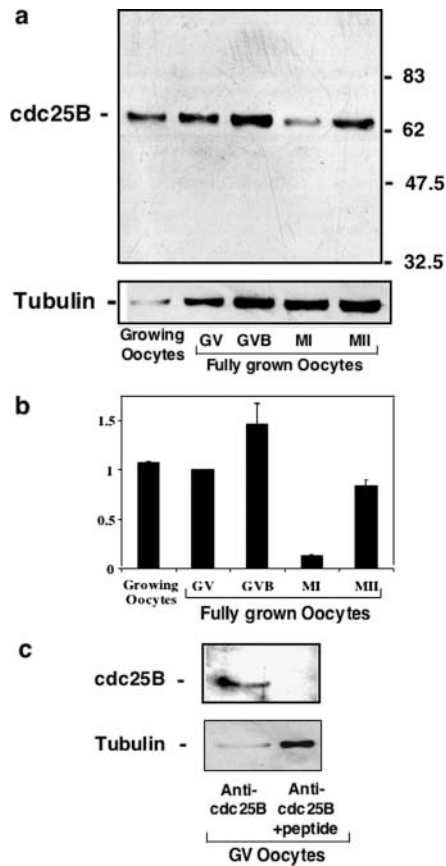
Figure 2 Expression of cdc25B in rat oocytes resuming meiosis: (a) oocytes at different stages of meiosis were extracted and their proteins separated by SDS-PAGE (350 oocytes/lane) followed by immunoblotting with anti-cdc25B antibodies. The results of one representative experiment are presented; (b) quantitation of four independent experiments with similar results; (c) incubation of the antibody with its relevant peptide eliminated immunoreactivity indicating specificity towards cdc25B.
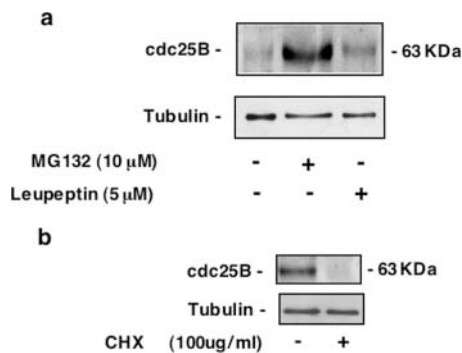

Figure 3 Regulation of cdc25B expression in rat oocyte. Oocytes incubated for the indicated periods of time, were extracted and their proteins separated by SDS-PAGE (400 oocytes/lane) followed by immunoblotting with anti-cdc25B antibodies: (a) oocytes were incubated for 8 h with MG132, a specific proteasomal inhibitor, or leupeptin, a lysosomal inhibitor; (b) oocytes were incubated for 8 h to reach MI. CHX was added and the oocytes were further incubated for additional 16 h. The experiments were repeated three times; the results of one representative experiment are presented.

Translational regulation of cdc25B involves cytoplasmic polyadenylation

In order to test whether the elevation of cdc25B at MII, is regulated by translation, oocytes were incubated for 8 h to allow their progress to MI of meiosis. At this time point, the protein synthesis inhibitor, cyclohexamide (CHX) was added, and the oocytes were examined for the presence of cdc25B after an additional 20 h. As shown in Fig. 3b, CHX blocked the reappearance of cdc25B at MII. This finding raised the possibility that the accumulation of cdc25B at MII can represent its translational regulation.

Fully grown oocytes that resume meiosis are transcriptionally dormant. These oocytes contain a pool of mRNA, some of which will be eventually translated. The translation of a selective preexiting mRNA is controlled by elongation of its polyA tail (Colgan & Manley 1997). For further confirmation of translational regulation, oocytes at different stages of meiosis were tested for their cdc25B mRNA polyadenylation. This experiment indeed revealed changes in the length of the cdc25B mRNA polyA tail that coincided with the modifications in the abundance of its protein product demonstrated in this study. Specifically, the GV oocytes possessed a short polyA tail that underwent elongation at GVB. The polyA tail of cdc25B mRNA was short again at MI, with another round of its elongation demonstrated at MII (Fig. 4a). No polyadenylation product was detected in the control samples that were not subjected to RT (data not shown). In order to prove specificity, the polyA samples were subjected to digestion by the restriction enzyme BtsI. The decrease in the size of the reaction product after digestion by 60 bp, confirmed its identity as cdc25B mRNA (Fig. 4b).

As previously mentioned by Colgan & Manley (1997), elongation of the polyA tail of a selective mRNA in oocytes is a perquisite for its translation. In order to confirm that cytoplasmic polyadenylation of the cdc25B, mRNA indeed mediates the synthesis of its corresponding protein, we interfered with this reaction by the use of cordycepin (Rose *et al.* 1977). As shown in Fig. 4c, oocytes incubated in the presence of cordycepin failed to exhibit the elevated expression of cdc25B demonstrated in oocytes incubated in the absence of this inhibitor. This experiment demonstrates the absolute dependency of cdc25B accumulation on cytoplasmic polyadenylation further confirming our suggestion that the expression of this protein is regulated at the level of translation.

Translocation of cdc25B in oocytes resuming meiosis

Availability of cdc25B can serve as one mechanism for the control of its activity during meiosis. An additional mode of regulation of the catalytic activity of this enzyme could be possibly provided by its subcellular
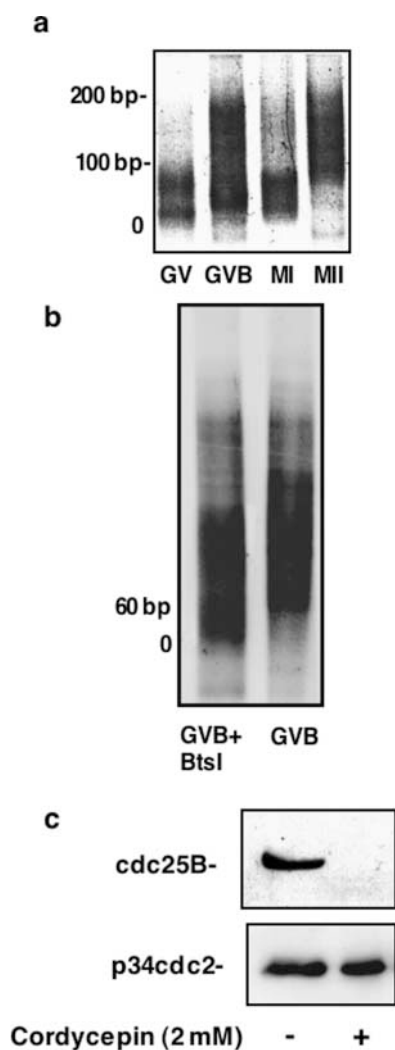
Figure 4 Polyadenylation of cdc25B mRNA in rat oocytes resuming meiosis: (a) total RNA was extracted from oocytes at different stages of meiosis, and the PAT, described in Materials and Methods, was preformed; (b) total RNA was extracted from oocytes at GVB stage, and the PAT test was preformed. The polyA samples were subjected to digestion by the restriction enzyme BtsI; (c) oocytes were incubated for 8 h to reach MI. Cordycepin was added and the oocytes were further incubated for an additional 16 h. The oocytes were subjected to Western blot analysis as described in Fig. 3. The experiment was repeated three times; the results of one representative experiment are presented.

translocation. To examine this possibility changes in cdc25B localization during resumption of meiosis were monitored by immunostaining. We found that in meiotically arrested oocytes (Fig. 5a A, B) cdc25B is present in the cytoplasm and is absent in the nucleus (Fig. 5a C). After GVB (Fig. 5a E, F) cdc25B migrates towards the condensed chromosomes (Fig. 5a G). Oocytes arrested at MII (Fig. 5a I, J) exhibit low cytoplasmic abundance of cdc25B and co-localization of this protein with the MII spindle apparatus (Fig. 5a K). Preincubation of the antibody with the relevant peptide,

abolished the staining, indicating its specificity towards cdc25B (Fig. 5b).

Discussion

Our findings demonstrate an inhibitory effect of neutralizing cdc25B antibodies on reinitiation of meiosis, further presenting changes in cdc25B localization in rat oocytes resuming meiosis. We also reveal a translationally regulated periodical accumulation of cdc25B that corresponds with the oscillatory pattern of MPF activity throughout meiosis. We show herein that cdc25B translation is mediated by polyadenylation of its mRNA. Taken together, our findings confirm the central role of cdc25B in the control of meiosis in rodent oocytes. They further suggest that temporally and spatially regulated–availability of this phosphatase may coordinate its activity.

The first clue linking cdc25 to the control of cell cycle was provided by observations that a G2-arrest in fission yeast is caused by cdc25$^-$ mutations (Fantes 1981). Later studies in fission yeast showed that the level of cdc25 mRNA oscillates through the cell cycle, reaching maximum at mitosis (Moreno *et al.* 1989, Ducommun *et al.* 1990). These studies raised the idea that periodic accumulation of the cdc25 protein is one of the rate-limiting processes regulating the transition into M-phase. Other studies demonstrated that inactivation of cdc25 results in a late G2-arrest that is accompanied by p34cdc2 phosphorylation (Gould & Nurse 1989). These and later demonstrations that cdc25 function is not required in strains expressing non-phosphorylatable p34cdc2 (Gould *et al.* 1990) were consistent with the idea that cdc25 functions to dephosphorylate this kinase.

The information generated in yeast on the role of cdc25 in regulating mitosis has been confirmed for the control of cell division in higher organisms (Sadhu *et al.* 1990, Gautier *et al.* 1991). Furthermore, studies in *Xenopus* oocytes disclosed a similar function for this enzyme in the control of meiosis (Izumi *et al.* 1992, Jessus & Beach 1992, Rime *et al.* 1994). On the other hand, hardly any information has been generated regarding cdc25 in mammalian oocytes. Since mammals express multiple cdc25 genes (Sadhu *et al.* 1990, Galaktionov & Beach 1991, Millar *et al.* 1991, Nagata *et al.* 1991) the identity of the specific isoform responsible for p34cdc2 dephosphorylation in mammalian oocytes resuming meiosis raised an additional point of interest.

As mentioned previously, it was commonly accepted that in mammals it is the cdc25C isoform that regulates the exit from G2 and entry into M-phase of the cell cycle (Gautier *et al.* 1991, Millar *et al.* 1991). Since reinitiation of meiosis represents a G2- to M-transition, the logical assumption was that resumption of meiosis in mammalian oocytes is subjected to regulation by cdc25C. However, the observation that knockout mice, lacking
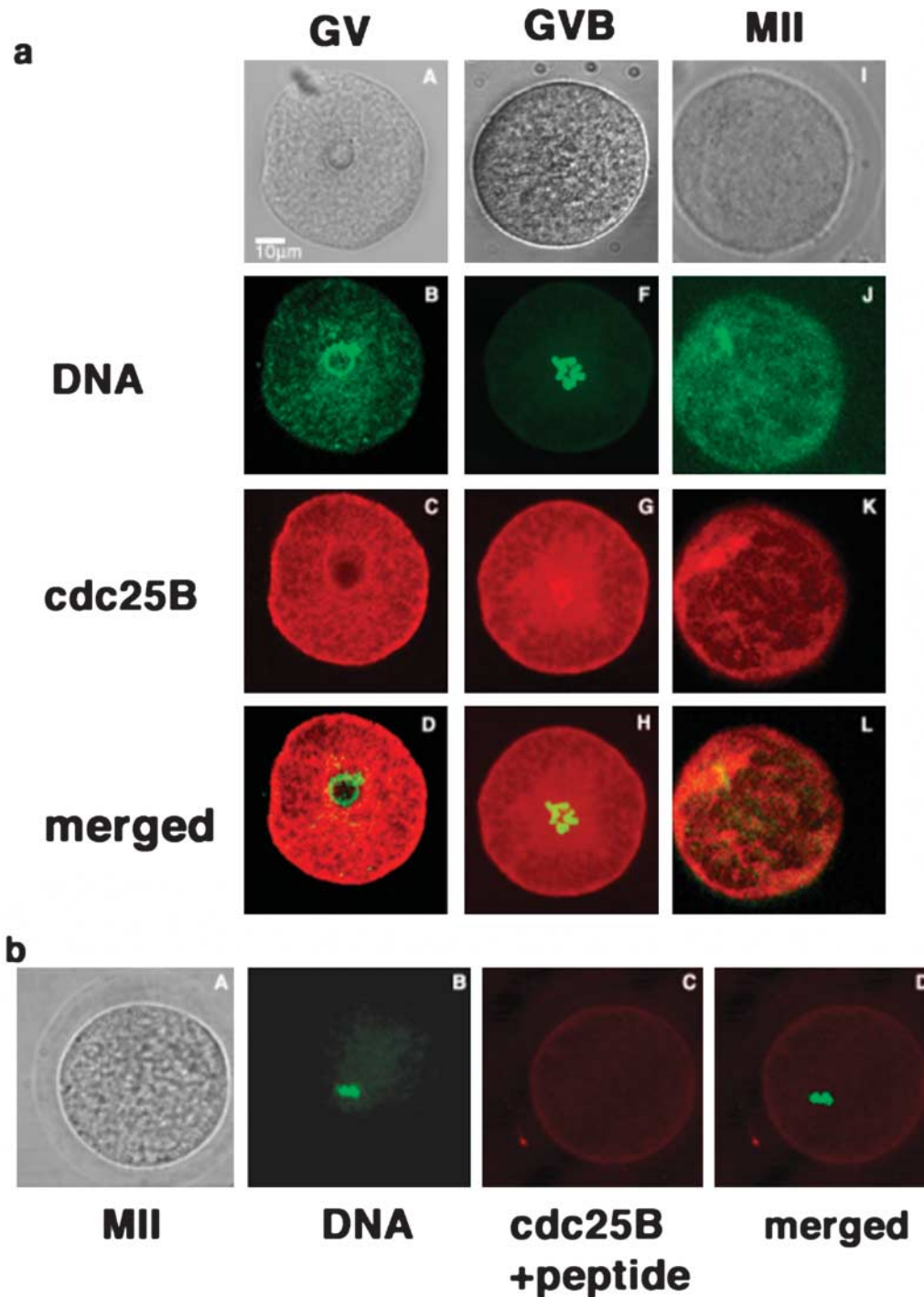
Figure 5 (a) Translocation of cdc25B in rat oocytes resuming meiosis. Spontaneously maturing oocytes fixed at various stages of meiosis, were immunoreacted with anti-cdc25B antibodies (red) and exposed to syto 13 for DNA staining (green). A meiotically arrested oocyte (A) characterized by the presence of GV (B) showing cytoplasmatic localization of cdc25B (C) and its absence in the nucleus. An oocyte incubated for 6 h (E), the nuclear membrane of which has disassembled and the chromosomes are condensed (F) shows migration of cdc25B to the chromosomes (G). An oocyte arrested at MII (I), with the chromosomes aligned on the metaphase plate (J), displays translocation of cdc25B to the spindle apparatus (K). The staining for cdc25C (red) and DNA (green) visualized together (D, H, L); (b) Preincubation of the cdc25B antibody with the relevant peptide abolished the staining in MII oocytes, indicating its specificity towards cdc25B (A–C).

cdc25C, were fertile (Chen *et al.* 2001) denied a role for this isoform in regulation of meiosis.

The candidacy of cdc25B in regulating reinitiation of meiosis was initially postulated by demonstrations that microinjection of this isoform into *Xenopus* oocytes induced GVB (Powers *et al.* 2000). A more recent study showing that oocytes of female mice lacking cdc25B fail to activate MPF and remain meiotically arrested, confirmed this idea (Lincoln *et al.* 2002). The inhibitory effect of anti-cdc25B and not anti-cdc25C antibodies on reinitiation of meiosis demonstrated herein agrees with the notion that it is the B isoform of the cdc25 phosphatase family that plays a role in reinitiation of meiosis in rat oocytes. These results further suggest that cdc25B and cdc25C do not have redundant roles in regulating meiosis resumption. In fact, some supporting evidence of the role of cdc25B in entry into M-phase has also been generated in mitotically dividing cells. These studies demonstrated that injection of specific antibodies into human fibroblasts or overexpression of cdc25B mutants in HeLa cells inhibit entry into mitosis (Gabrielli *et al.* 1996, Lammer *et al.* 1998). Another study showed that overexpression of cdc25B in HeLa cells causes premature transition into M-phase (Karlsson *et al.* 1999).

A complementary evidence for the role of cdc25B in regulation of oocyte meiosis is provided by the oscillatory pattern of its expression. Cell cycle associated modulation of the expression of cdc25B has been previously reported in mitosis (Russell & Nurse 1986, Gabrielli *et al.* 1996, 1997*a*, Lammer *et al.* 1998). However, regulation of cdc25 by modulating its availability in the meiotic cell cycle is demonstrated herein. We show that the abundance of this enzyme substantially increases at GVB, transiently decreases at MI and is elevated again at MII. This periodic accumulation of cdc25B nicely corresponds to the kinetics of MPF activity throughout meiosis (Doree *et al.* 1983, Hashimoto & Kishimoto 1986, Choi *et al.* 1991, Fulka *et al.* 1992, Josefsberg *et al.* 2003). It is therefore plausible that the activity of MPF in rat oocytes is controlled by the availability of cdc25B. Accumulation of cdc25B shown herein represents apparently *de novo* synthesis of the enzyme mediated by polyadenylation.

Fully grown oocytes that resume meiosis are transcriptionally dormant. Protein synthesis in these oocytes is enhanced by recruitment of pooled mRNA and its selective cytoplasmic polyadenylation (Colgan & Manley 1997). So far, mRNAs for Mos, tPA, cyclin A1 and B1 as well as wee1 (Huarte *et al.* 1987, Gebauer *et al.* 1994, Sheets *et al.* 1994, Stebbins-Boaz *et al.* 1996, Charlesworth *et al.* 2000) have been shown to undergo polyadenylation throughout meiosis. We demonstrate herein for the first time that similar to the above-mentioned proteins, cdc25B translation in oocytes resuming meiosis is also regulated by its mRNA polyadenylation. Identification of both CPE and hexanucleotide sequence in the cdc25B mRNA agrees with our

finding. Unlike the above-mentioned mRNAs, that exhibit an elongated polyA tail soon after reinitiation of meiosis that sustains throughout meiosis (Sheets *et al.* 1994, de Moor & Richter 1997, Lazar *et al.* 2002), polyadenylation of cdc25B mRNA and the subsequent accumulation of its protein product exhibits an oscillatory pattern.

It has been shown in *Xenopus* oocytes that polyadenylation of cyclin B and cyclin A mRNAs is Mos-dependent and that this effect of Mos is mediated by MPF (de Moor & Richter 1997). A later study in the rat revealed that Mos mRNA polyadenylation is subjected to negative regulation of cAMP and that this inhibitory effect is mediated by PKA (Lazar *et al.* 2002). A more recent report further demonstrated that Mos mRNA polyadenylation in rat oocytes will not take place in absence of an active MPF (Lazar *et al.* 2004). The mechanisms that regulate cdc25B expression during oocyte maturation remain to be elucidated.

The cdc25B mRNA polyadenylation followed by its translation in GVB oocytes, nicely corresponds with MPF activation at reinitiation of meiosis. However, the reduction in the amount of cdc25B at MI is somewhat unexpected, since inactivation of MPF upon completion of the first meiotic division that is subsequent to cyclin degradation, is not associated with p34cdc2 rephosphorylation (Choi *et al.* 1991, Goren & Dekel 1994). Furthermore, since reactivation of MPF upon the transition into MII is supposed to be solely dependent on cyclin synthesis, the second round of cdc25B mRNA polyadenylation was not anticipated. A clue for the possible role for cdc25B accumulation after completion of the first meiotic division is provided by a recent demonstration that co-expression of cdc25B and cyclin B in G1 cells drives the cells to enter mitosis without DNA replication (Karlsson *et al.* 1999). The concomitant elevation of cdc25B and cyclin B upon transition into MII may provide the mechanism that uncouples between cell division and DNA replication causing two sequential M-phases in meiosis. In addition, a previous study (Nakajo *et al.* 2000) claimed that wee1 decreases after reinitiation of meiosis and remains low until fertilization. Indeed, in the absence of wee1, there is no requirement for cdc25 to balance its effect on p34cdc2 phosphorylation. However, another report demonstrating that wee1 is present in oocytes resuming meiosis (Iwabuchi *et al.* 2000) disagrees with this idea further suggesting that accumulation of cdc25B may have a role in MPF reactivation at the entry into the second meiotic division.

The periodic pattern of cdc25B accumulation demonstrated herein in rat oocytes throughout meiosis is regulated by protein synthesis and apparently balanced by proteasomal degradation. Proteolysis of cdc25B that is brought about by the proteasome has been previously demonstrated (Baldin *et al.* 1997*a*, Cans *et al.* 1999). It has also been reported that at least in yeast cdc25 degradation is preceded by its ubiquitination (Nefsky & Beach 1996).

This study further identified pub1, a major ubiquitin ligase of cdc25, the disruption of which dramatically reduces ubiquitination of cdc25 further stimulating premature entry into mitosis. Along this line, we report herein that the KEN motif, a domain on the molecule required for ubiquitin-mediated proteolysis (Pfleger & Kirschner 2000) can be identified in the cdc25B molecule.

Regulation of cdc25B by synthesis and degradation could be modulated by subcellular localization restricting its function to a precise cellular location. In support of this idea, a cell cycle-dependent transport of cdc25B in and out the nucleus, has been demonstrated in somatic (Girard *et al.* 1992, Garner-Hamrick & Fisher 1998, Dalal *et al.* 1999, Davezac *et al.* 2000). Our immunocytochemical experiments revealed a restricted cytoplasmatic localization of cdc25B in G2-arrested oocytes. We further demonstrate herein that cdc25B co-localizes with the spindle apparatus, which is of most interest. It has been shown that cyclin B and cdc25B translocate to the nucleus in concert (Gabrielli *et al.* 1996, 1997*b*) and that the cytoplasmatic localization of cdc25B depends upon cyclin B (Karlsson *et al.* 1999). Since cyclin B has also been demonstrated to be associated with the meiotic spindle (our unpublished observations), these findings may represent compartmentalization of the enzyme and its substrate that increases the efficiency of its catalytic activity. Finally, another support to this idea is provided by a recent study demonstrating that cdc25B has a role in activating the cyclin B–Cdk1 complex on the centrosomes at the G2/M transition in Hela cells undergoing mitosis (Lindqvist *et al.* 2005). In summary, our findings not only confirm the role of cdc25B in rodent oocytes, but also present a unique mode of regulation of the activity of this phosphatase by temporal and spatial modulation of its availability.

Acknowledgements

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