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Maturation-Promoting Factor Governs Mitogen-Activated Protein Kinase Activation and Interphase Suppression During Meiosis of Rat Oocytes[1]

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ABSTRACT

Meiosis is a particular example of a cell cycle, characterized by two successive divisions without an intervening interphase. Resumption of meiosis in oocytes is associated with activation of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). The activity of MPF declines during the transition between the two meiotic divisions, whereas the activity of MAPK is sustained. Attempts to disclose the interplay between these key regulators of meiosis in both amphibian and mammalian oocytes generated contradictory results. Furthermore, the enzyme that governs the suppression of interphase in mammals is still unidentified. To our knowledge, we provide herein the first demonstration in a mammalian system that inhibition of MPF at reinitiation of meiosis abrogated Mos expression and MAPK activation. We also show that oocytes, in which reactivation of MPF at completion of the first telophase was prevented, exhibited an interphase nucleus with decondensed chromosomes. Inhibition of MAPK did not interfere with the progression to the second meiotic metaphase but, rather, resulted in parthenogenic activation. We conclude that in rat oocytes, MPF regulates MAPK activation and its timely reactivation prevents the oocytes from entering interphase.

cyclic adenosine monophosphate, meiosis, oocyte development

INTRODUCTION

Meiosis in mammalian oocytes starts during embryonic life and arrests around birth, at the first prophase. The arrested oocytes are characterized by the presence of an intact nucleus, known as the germinal vesicle (GV), and decondensed chromosomes. Meiosis is detained until puberty, when the oocyte is exposed to the midcycle surge of LH. Resumption of meiosis in vitro can be instigated spontaneously by separation of the oocyte from its surrounding follicle cells and is associated with a drop in intraoocyte concentrations of cAMP (reviewed in [1]).

Developmental capacity of in vitro-matured mouse oocytes that has been shown to be lower than that of oocytes matured in vivo in response to LH raised some concerns regarding the adequacy of the spontaneously matured oocyte system [2]. However, the above-mentioned as well as other studies [3, 4] demonstrated that modified culture conditions dramatically influence the meiotic response of the oocyte in vitro. These studies therefore suggest that under appropriate culture conditions, this widely used experimental model can generate results that are applicable in terms of understanding the physiological regulation of oocyte maturation.

Reinitiation of meiosis represents transition from G_2 to M phase in the cell cycle and is manifested by chromosome condensation, disintegration of the nuclear envelope (GV breakdown [GVB]), and spindle formation. The continuation of the meiotic process includes discharge of one set of homologous chromosomes into the first polar body (PBI) and arrest at metaphase of the second meiotic division (MII). The hallmarks of meiosis are the two consecutive M-phase divisions, in the absence of S phase, resulting in one haploid daughter cell. The period in between the two meiotic divisions is defined as interkinesis, during which the oocyte is secured from entering interphase.

Resumption of meiosis is regulated by the maturation-promoting factor (MPF), which was originally identified as an activity that triggers reinitiation of meiosis in frog oocytes [5] and was subsequently observed in a variety of meiotically and mitotically dividing cells. Purification of MPF disclosed a heterodimer composed of a catalytic, 34-kDa serine/threonine kinase, designated p34cdc2, and a regulatory cyclin B (reviewed in [6]). Other enzymes that have been shown to be activated during meiosis are the 42- and 44-kDa isoforms of the mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs, reviewed in [7, 8]). Activation of these serine/threonine kinases is mediated via phosphorylation by an upstream, 45-kDa MAPK kinase termed MEK. In oocytes, MEK is activated by a germ cell-exclusive kinase, Mos. The Mos/MAPK pathway has been designated as the cell cytostatic factor, which is responsible for the second metaphase arrest [5, 9].

Attempts to study the interplay between MPF and MAPK in *Xenopus* oocytes included biochemical manipulation of either enzyme [10, 11]. Some of these studies suggested that the presence of an active Mos/MAPK pathway is a prerequisite for MPF activation [12–14], whereas other reports demonstrated the converse hierarchy [15, 16]. No controversy has occurred regarding the role of MAPK as the factor governing interkinesis in amphibian oocytes [17, 18].

Early biochemical and genetic manipulation of the Mos/MAPK pathway in the mouse suggested that in mammalian oocytes, Mos/MAPK activation is necessary for reinitiation of meiosis [19–21]. However, later demonstrations that MPF activation in the mouse occurs before MAPK activation [22, 23] disagree with this conclusion. The fact that MPF regulation is not mediated by Mos/MAPK is further emphasized by oocytes derived from mos knockout mice, which display a normal pattern of MPF activation [24–26]. Moreover, unlike previous reports that showed Mos/MAPK

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activation is necessary for suppression of interphase after completion of the first telophase [27, 28], oocytes of these mutant mice exhibit proper interkinesis despite the lack of an active MAPK [29, 30].

Taken together, the published evidence regarding the interplay between MPF and MAPK at reinitiation of meiosis in mammals is contradictory. Additionally, the factor governing interphase suppression during interkinesis is still undefined. Our hypothesis is that in the rat, MAPK activation is governed by MPF, and that MPF activity mediates the suppression of interphase between the two meiotic divisions. To test our hypothesis, we investigated the implications of MPF inhibition during several stages of meiosis: 1) prevention of MPF activation at the onset of meiosis, 2) inhibition of the enzyme at metaphase of the first meiotic division (MI), and 3) impediment of MPF reactivation at the entry to MII. We present, to our knowledge, the first results in a mammalian oocyte system proposing that MPF facilitates MAPK activation through regulation of Mos expression. Furthermore, we provide evidence suggesting a role for MPF in preventing the oocyte from entry into interphase between the two rounds of meiosis.

MATERIALS AND METHODS

Reagents and Antibodies

Leibovitz L-15 tissue culture medium was purchased from Gibco BRL (Paisley, Scotland). Antibiotics were purchased from Bio-Lab Ltd. (Jerusalem, Israel). The PD098059 was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Roscovitine was purchased from Calbiochem (La Jolla, CA). Leupeptin, hyaluronidase, isobutylmethylxanthine (IBMX), dibutyryl cyclic AMP (dbcAMP), 3-[*N*-morpholino]propane-sulfonic acid (MOPS), histone H1 (type III-S), 4′,6′-diamidino-2-phenylindole (DAPI), antibodies for the double antibody-system detection of the activation state of MAPK, anti-β-tubulin monoclonal antibodies, and fetal bovine serum were purchased from Sigma (St. Louis, MO). Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Jackson (San Francisco, CA). [γ-^{32}P]Adenosine 5′-triphosphate (ATP; 3000 Ci/mmol) and enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Amersham (Buckinghamshire, U.K.). Affi-Prep Protein A-agarose beads were purchased from Bio-Rad (Hercules, CA). Rabbit sera raised against the carboxy terminus of p42 MAPK of rat origin that cross-reacts with p44 MAPK (C-14), goat sera raised against Mos, and rabbit sera raised against the carboxy terminus of Cdc2 p34 of human origin (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against cyclin B1 (V152) were purchased form DAKO (Carpinteria, CA).

Animals

Sexually immature, 23- to 25-day-old, female Wistar rats (Harlan, Rehovot, Israel) were injected s.c. with 8 IU of eCG (Sanofi Sante Nutrition Animale, France) in 0.1 ml of 0.9% NaCl for induction of follicular development. Prophase-arrested oocytes were recovered from the ovaries 48 h after injection. Postovulatory, MII-arrested oocytes were obtained from the oviducts of the above-described rats injected with 5 IU of hCG (Pregnyl; N.V. Organon Oss, Holland) in 0.1 ml of 0.9% NaCl 48–52 h after eCG administration to induce ovulation. The rats were killed by cervical dislocation 24 h after the hCG injection.

The present study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD).

Oocyte Recovery and Culture

The prophase-arrested oocytes were isolated into Leibovitz L-15 tissue-culture medium, supplemented with 5% fetal bovine serum, 100 IU/ml of penicillin, 50 μg/ml of streptomycin, and 0.25 μg/ml of fungizone. Cumulus cells were removed by incubation with a mild-chelating agent (3 mM EDTA, pH 8.0, in L-15 at 37°C for 30 min), followed by repetitive pipetting. Denuded oocytes were incubated in a 37°C, humidified incubator to allow their spontaneous maturation. At the end of incubation, the oocytes were analyzed for maturation by differential interference-contrast microscopy. The presence of GV was used to classify oocytes as meiotically arrested at the prophase stage. Resumption of meiosis, which was indicated by GVB, occurred spontaneously in oocytes incubated for 4 h after their isolation from ovarian follicles. To prevent GVB, oocytes were incubated either in 0.2 mM of the cAMP phosphodiesterase-inhibitor IBMX or a combination of 2 mM dbcAMP and 0.02 mM IBMX [31]. The PBI was emitted at 10–12 h of incubation in inhibitor-free medium, and following an overnight incubation, the oocytes were arrested at metaphase of MII.

The postovulatory oocytes were recovered from oviducts into PBS. The cumulus cells of these oocytes were removed enzymatically by hyaluronidase (1 mg/ml, 10 min, 37°C), followed by repetitive pipetting.

Histone H1 Kinase Activity

The MPF activity was determined by the histone H1 kinase assay that is routinely used for monitoring p34cdc2 kinase. Histone H1 kinase activity was measured in lysates of 50 oocytes, prepared by freezing and thawing in 10 μl of kinase buffer (15 mM MOPS, 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 10 μg/ml of PKI, a cAMP-dependent protein kinase inhibitor peptide), followed by centrifugation (10 000 rpm) for 10 min at 4°C. Kinase reactions were initiated by the addition of 10 μl of substrate buffer (2 mg/ml of histone H1, 2 mM dithiothreitol [DTT], and 5 μCi [γ-^{32}P]ATP), and the reactions were carried out at 30°C for 30 min. Kinase reaction products were subjected to SDS-PAGE, followed by overnight washing in 1% tetrasodiumdiphosphate-decahydrate, 5% trichloroacetic acid, and autoradiography. Densitometric analysis was performed utilizing the Fujix BAS1000 PhosphorImager supported by MacBas software (Fujix, Tokyo, Japan).

MAPK Phosphorylation

The activation state of MAPK was determined by Western blot analysis utilizing anti-double phosphorylated MAPK monoclonal antibody that detects the phosphorylated, active MAPK and a rabbit polyclonal serum that detects the total amount of MAPK [32].

At the end of the specified incubation time, 50 oocytes were lysed in 10 μl of homogenization buffer (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM Na-orthovanadate, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 2 μg/ml of pepstatin, and 1 mM DTT) and subjected to Western blot analysis. The relevant HRP-conjugated secondary antibodies were used, and immunoreactive bands were detected by ECL reagents. Densitometric analysis was performed utilizing the Pdi 420oe densitometer supported by Quantity One software (Pdi, Huntington Station, NY).

Myelin Basic Protein Kinase Activity

The MAPK activity was determined by a myelin basic protein (MBP) kinase assay that is routinely used for monitoring MAPK activity. Taking into account that postovulatory oocytes contain an MBP kinase activity that is independent of MAPK [22], to ascertain its specificity the assay was performed on immunoprecipitates of MAPK. Postovulatory oocytes (200 per sample) were lysed in 50 μl of homogenization buffer and subjected to three cycles of freezing and thawing, followed by centrifugation (10 000 rpm) for 10 min at 4°C. The antibody-protein A complex was prepared by incubation overnight at 4°C of 1.25 μl of anti-MAPK antibody with 15 μl of Affi-Prep Protein A-agarose beads at a final volume of 500 μl. The beads were washed three times in buffer A (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, and 1 mM Na-orthovanadate), after which the lysates were added at a final volume of 200 μl of homogenization buffer and further incubated for 2 h at 4°C. At the end of the precipitation, the beads were washed once with RIPA buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, and 10 μg/ml of leupeptin), twice with 0.5 M LiCl and 0.1 M Tris (pH 8.0), and once with buffer A. Kinase reactions were initiated by the addition of 30 μl of substrate buffer (2 mg/ml of MBP, 5 μCi [γ-^{32}P]ATP, 25 mM β-glycerophosphate, 5 mM DTT, 1.3 mM EGTA, 50 mM Na-orthovanadate, 10 mM $MgCl_2$, 100 μM ATP, 10 μM calmiolozolium, 830 μg/ml of BSA, and 2 μM PKI), and the reactions were carried out at 30°C for 30 min. Kinase reaction products were subjected to SDS-PAGE and autoradiography. Densitometric analysis was performed utilizing the Fujix BAS1000 PhosphorImager supported by MacBas software.

Western Blot Analysis

Oocytes were lysed in homogenization buffer and subjected to Western blot analysis. Rabbit antisera against p34cdc2 (1:500 dilution, v/v), monoclonal antibodies against cyclin B1 (1:750), goat antisera against mos (1:500), and their respective HRP-conjugated secondary antibodies were used, and immunoreactive bands were detected by ECL reagents. Densitometric analysis was performed utilizing the Pdi 420oe densitometer supported by Quantity One software.

Fluorescent DNA Staining

Oocytes were fixed by 3% paraformaldehyde (20 min, room temperature), washed three times with GB-PBS (10 mM glycine and 10 mg/ml of BSA in PBS), followed by permeabilization with 1% Triton X-100 (4 min, room temperature), washed three times with GB-PBS, and placed into DAPI (1:400) for 1 h at room temperature. Following three washes with GB-PBS, the oocytes in 50% glycerol/PBS were mounted on silicon-coated glass slides and covered by coverslips resting on a silicone ring containing 100-μm glass beads that served as spacers. The oocytes were visualized by both phase-contrast and fluorescent microscopy using an Optiphot-2 microscope (Nikon, Tokyo, Japan) equipped with BP546/455 filters.

RESULTS

cAMP Inhibits MPF Activation and Cyclin B1 Expression

Our initial experiment confirmed the activation pattern of MPF in rat oocytes resuming meiosis spontaneously, and we further analyzed the effect of cAMP in this system. Similar to previous studies with rodent oocytes [33, 34], we show that prophase-arrested oocytes display a low MPF activity that is initially increased at 2 h after their isolation from the ovarian follicle (Fig. 1A). This early MPF activation temporally corresponds to the decrease in cAMP concentrations demonstrated by us previously in rat oocytes resuming meiosis [35]. This correlation agrees with the inhibitory effect of cAMP on MPF activation at reinitiation of meiosis demonstrated herein (Fig. 1A). Quite interestingly, cAMP had no influence on MPF reactivation on the transition to MII (data not shown).

The inhibitory effect of cAMP on the initial phase of MPF activation (Fig. 1A) can be attributed to the previously demonstrated interference of this cyclic nucleotide with p34cdc2 dephosphorylation [33, 36]. In the present study, we found that incubation under conditions that elevate intraoocyte concentrations of cAMP also resulted in repression of cyclin B1 expression. (Fig. 1B). Thus, in addition to maintenance of the MPF in its inactive form, the cAMP negative action is also elicited at the level of cyclin availability.

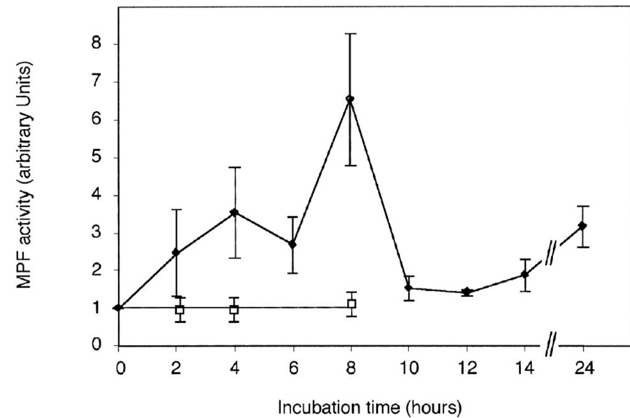
MPF Controls MAPK Activation and Mos Expression

It is well established in mammals that MPF activation is independent of MAPK [24–26]. However, to our knowledge, the possibility that MPF governs MAPK activation has not been investigated in mammalian oocytes. To test the hypothesis that MPF is a regulator of the MAPK-signaling cascade, the activation of MAPK was assessed in the absence of an active MPF. To interfere with MPF activity, we utilized roscovitine, a purine analogue that selectively inhibits p34cdc2 kinase [37, 38]. Our initial experiment was directed at characterizing the effect of roscovitine in our experimental system. These experiments revealed that a range of roscovitine concentrations similar to those that inhibited histone H1 kinase activity in rat oocytes (Fig. 2A) also prevented their resumption of meiosis (Fig. 2B). Incubation of the oocytes with roscovitine for 8 h, the time that corresponds to the initial MAPK activation [39], resulted in a dose-dependent inhibition on phosphorylation of both p44 MAPK and p42 MAPK (Fig. 3A). The repressed MAPK kinase activation by inhibition of MPF may support our hypothesis regarding the sequential order of MPF and MAPK activation. However, it could possibly represent direct inhibition of MAPK by roscovitine.

To resolve the issue of roscovitine specificity, we analyzed its direct effect in vitro on immunoprecipitated MAPK from lysates of MII-arrested oocytes that express a high level of MAPK activity. Furthermore, unlike the previous experiments that employed Western blot analysis determining the abundance of the phosphorylated protein, the present experiment tested the catalytic activity of the enzyme. This last strategy takes into account the possible occupation of the ATP-binding pocket by roscovitine and the subsequent inhibition of a phosphorylated enzyme. A dose of roscovitine that was maximally effective in inhibiting MAPK activity in whole-oocyte lysates had no effect in this experiment, confirming the idea of no direct inhibition by this drug (Fig. 3B).

To pinpoint the timing of the MPF-dependent event that regulates MAPK activation, oocytes were incubated in inhibitor-free medium for either 1, 2, or 3 h and then placed into roscovitine. The oocytes were lysed at the end of an 8-h incubation period, and the activation state of MAPK was assessed. Figure 3C depicts that unlike oocytes continuously incubated in the presence of roscovitine, those oo-

A



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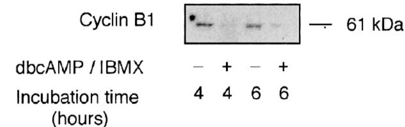

FIG. 1. cAMP inhibits MPF activation and cyclin B1 expression. **A**) Rat oocytes (50 oocytes/sample) were extracted at the various time points after their incubation with (□) or without (◆) IBMX (0.2 mM) and assayed for histone H1 kinase activity. The values (mean ± SEM) of the fold-increase in histone H1 kinase activity at each time point over the activity at time 0 are presented in pooled results from at least three individual experiments. **B**) Oocytes (100 oocytes/lane) were incubated in the absence or presence of dbcAMP (2 mM) and IBMX (0.02 mM) for 4 and 6 h. The oocytes were then extracted and subjected to SDS-PAGE followed by immunoblotting against cyclin B1. A representative result of at least three individual experiments is presented.

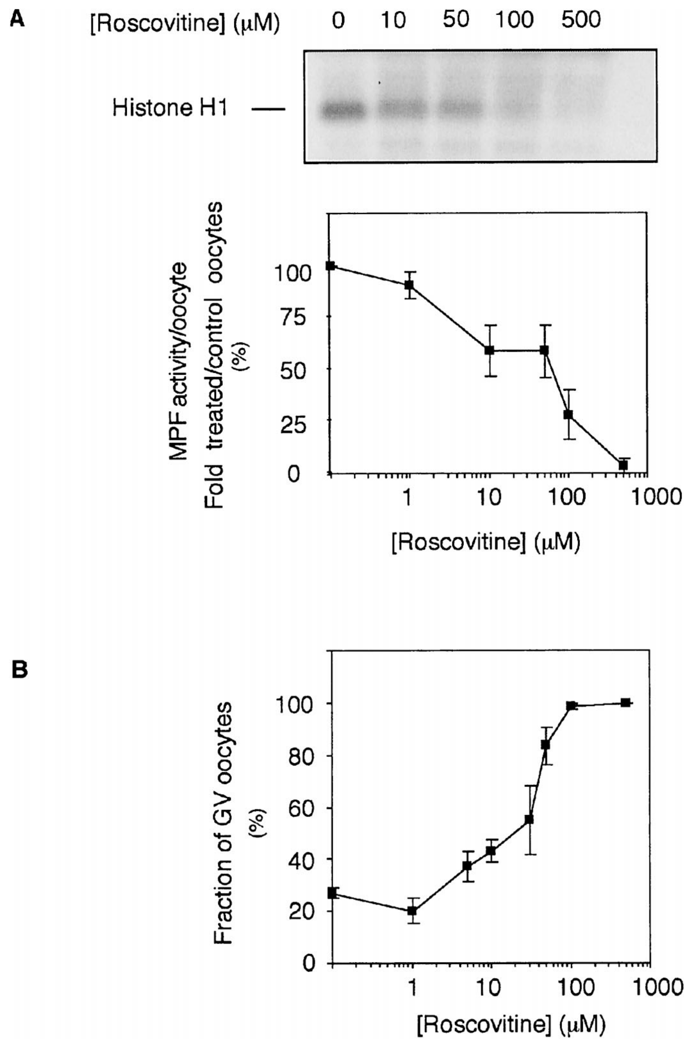

FIG. 2. Characterization of the effect of roscovitine on rat oocytes. **A**) Oocytes incubated for 8 h in increasing concentration of roscovitine were extracted and assayed for histone H1 kinase activity. The results of one representative experiment are presented in the upper panel. The lower panel depicts the fold-increase in kinase activity of treated over control oocytes. The values (means ± SEM) of pooled results from three individual experiments are presented. **B**) Oocytes were incubated for 8 h with roscovitine and monitored morphologically by differential interference-contrast microscopy. The fraction of oocytes that displayed a germinal vesicle (GV) is presented. The means of at least eight different experiments (minimum, 350 oocytes for each experimental point) are presented along with their SEMs.

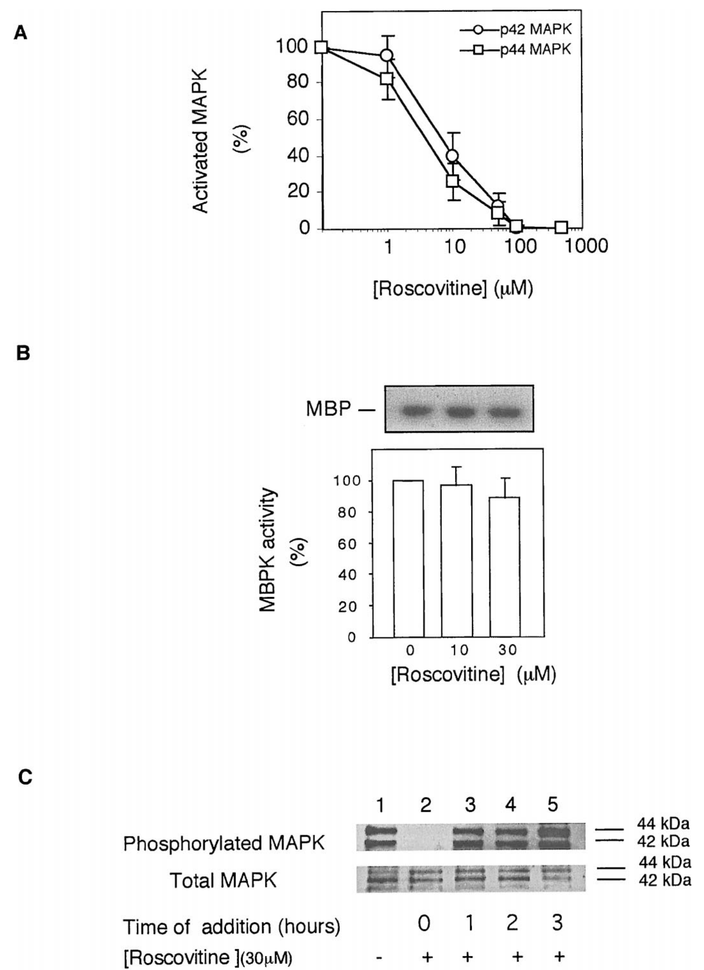

FIG. 3. Effect of MPF inhibition on MAPK activation. **A**) Oocytes (50 oocytes/lane) were incubated in increasing concentrations of roscovitine for 8 h. The oocytes were then extracted and subjected to SDS-PAGE followed by immunoblotting against phosphorylated and total MAPK. The ratio of phosphorylated MAPK normalized over total MAPK of treated over control oocytes is presented. Values (mean ± SEM) of pooled results from at least three individual experiments are presented. **B**) Postovulatory oocytes (200 oocytes/sample) were immunoprecipitated with anti-MAPK antibodies. Immunoprecipitated MAPK was assayed for MBP kinase activity in vitro in the absence or presence of roscovitine. The results of one representative experiment are presented in the upper panel. The lower panel depicts the ratio of MBP kinase activity of treated over control immunoprecipitates. Values (mean ± SEM) of pooled results from three individual experiments are presented. **C**) Oocytes were incubated for a total of 8 h under the following conditions: in inhibitor-free medium (lane 1), in the continuous presence of 30 μM roscovitine (lane 2), and in inhibitor-free medium for 1, 2, and 3 h and than transferred to 30 μM roscovitine for the rest of the incubation time (lanes 3–5, respectively). The oocytes were then extracted and subjected to SDS-PAGE followed by immunoblotting against phosphorylated and total MAPK. A representative result of at least three individual experiments is presented.

cytes given even 1 h of inhibitor-free medium expressed an activated MAPK. This finding not only confirms the role of MPF in regulating MAPK but further identifies the first hour of reinitiation of meiosis as the time during which oocytes are committed to activate MAPK.

As already mentioned, inhibition of MPF activation immediately upon resumption of meiosis resulted in repression of MAPK phosphorylation (Fig. 4A, lane 2). On the other hand, the presence of a phosphorylated MAPK was not influenced in oocytes exposed to roscovitine at the onset of MII (Fig. 4A, lanes 3 and 4). These findings suggest that it is not the activity but, rather, the activation of MAPK that is subjected to MPF regulation. Because MAPK activation in oocytes is regulated by the mos mRNA protooncogene product, we further examined the possible influence of MPF on the expression of Mos. This experiment reveals that whereas control oocytes incubated for 8 h expressed high levels of Mos, roscovitine-treated oocytes had undetectable levels of the protein at the same time point (Fig. 4B). Participation of Mos expression in mediating the effect of MPF on MAPK activation may explain the 5-h time interval that elapses between the commitment of oocytes to activate MAPK demonstrated in the previous experiment and the actual activation of this enzyme that is not detected before 6 h of incubation [39].

To examine whether the activation state of p34cdc2 may reflect regulation of its downstream effectors, we analyzed roscovitine-treated oocytes for the state of p34cdc2 phosphorylation. Previous studies in mice [33] and rats [36]

established the expression profile of p34cdc2, in which three different migrating forms of the enzyme were present in conjunction with its phosphorylation. The phosphorylated forms of p34cdc2 represent the inactive enzyme, whereas their disappearance directly correlates to MPF activation. We show herein that MII-arrested oocytes display an active, nonphosphorylated form of p34cdc2, whereas oocytes that were exposed to roscovitine immediately after PBI extrusion possess a phosphorylated p34cdc2 (Fig. 4C), reflecting inactivation of the enzyme. This inhibitory rephosphorylation of p34cdc2 possibly relates to the known autocatalytic loop of MPF activation [40].

MPF Activity Is Required for Suppression of Interphase

The outcome of p34cdc2 inhibition was further inspected at the transition period between the two meiotic divisions. In this experiment, freshly isolated oocytes, incubated in an inhibitor-free medium for 8–10 h, were individually selected before or immediately after PBI extrusion and then further incubated in the presence or absence of roscovitine for an additional 6 h. Similar to our previous results [39], 60% of the oocytes incubated continuously in inhibitor-free medium extruded PBI. Inhibition of MPF activity before the emission of PBI resulted in formation of a well-defined nucleus in 47.23% ± 9.9% of these oocytes (n = 417, mean ± SEM). None of these oocytes extruded PBI. Inhibition of MPF reactivation consequent to completion of MI prevented entry into the second metaphase, misleading the oocytes into interphase. This effect was manifested by the formation of a nucleus within 65.0% ± 5.3% of oocytes that extruded PBI (n = 302, mean ± SEM) (Fig. 5A2). Nuclear formation was not observed in oocytes with PBI that were incubated in an inhibitor-free medium.

The chromosomal status of the roscovitine-treated oocytes was verified by fluorescent staining of their DNA. Control, prophase-arrested oocytes displayed a typical perinuclear staining of the GV (Fig. 5B2), whereas oocytes that were treated with roscovitine before PBI extrusion displayed partially decondensed chromatin inside the nucleus (Fig. 5C2). As the meiotic division progresses, control oocytes in which PBI has been extruded exhibited the two sets of segregated, homologous chromosomes, inside the oocyte and at the PBI (Fig. 5D2). In contrast, the oocytes treated with roscovitine after PBI extrusion displayed decondensed DNA within the nucleus (Fig. 5E2).

MAPK Is Required for Second Metaphase Arrest

Our experiments directed at the control of MPF on Mos expression, MAPK activation, and interphase suppression were followed by examination of the roles of MAPK in regulation of meiosis in rat oocytes. For that purpose, prophase-arrested oocytes were incubated in the absence or presence of PD098059, a potent inhibitor of MEK, the direct upstream regulator of MAPK [41]. As anticipated, prevention of MAPK activation by PD098059 (Fig. 6A) had no effect on the initiation and completion of MI, as indicated by GVB and PBI formation (Table 1). However, in the absence of an active MAPK, the oocytes did not arrest at metaphase II, and a relatively large fraction of them (23.7%, n = 356) emitted the second polar body (PBII) (Table 1 and Fig. 6B3). Fluorescent staining of DNA in control oocytes detected the presence of two stained aggregates relating to the two sets of homologous chromosomes, one within the ooplasm and the other inside the PBI (Fig. 6B2). In contrast, PD098059-treated oocytes display the

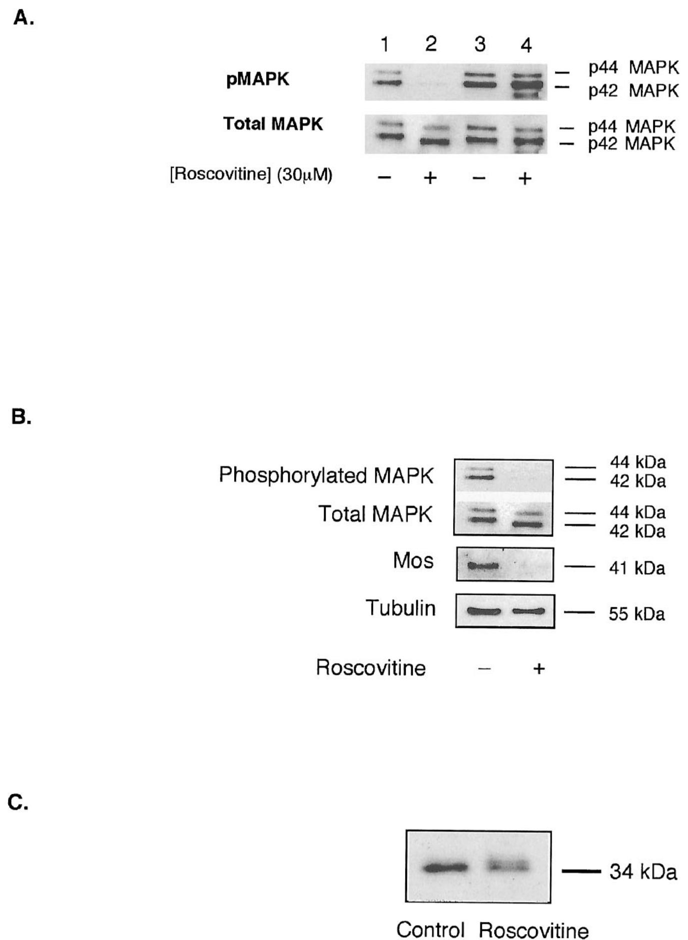

FIG. 4. Inhibition of MPF activation prevents Mos expression and results in p34cdc2 rephosphorylation. **A**) Oocytes were incubated for 8 h (lanes 1 and 2) or 16 h (lanes 3 and 4) under the following conditions: in inhibitor-free media for 8 h (lane 1), in the continuous presence of 30 μM roscovitine for 8 h (lane 2), and individually selected immediately after PBI extrusion and incubated in either inhibitor-free medium (lane 3) or the presence of 30 μM roscovitine (lane 4) for an additional 8 h. At the end of the incubation period, the oocytes were extracted and subjected to SDS-PAGE followed by immunoblotting against phosphorylated and total MAPK. A representative result of at least three individual experiments is presented. **B**) Oocytes were incubated for 8 h in the presence of roscovitine (100 μM). The oocytes were then extracted and subjected to SDS-PAGE followed by immunoblotting against phosphorylated and total MAPK (50 oocytes/lane), Mos and β-tubulin (500 oocytes/lane). A representative result of at least three individual experiments is presented. **C**) Oocytes (100 oocytes/lane) were individually selected immediately after PBI extrusion and incubated in 30 μM roscovitine for an additional 12 h. At the end of the incubation period, the oocytes were extracted and subjected to SDS-PAGE followed by immunoblotting against p34cdc2. A representative result of at least three individual experiments is presented.

presence of three chromosomal aggregates: One such aggregate localized in the ooplasm, and the other two localized in PBI and PBII (Fig. 6B4). The consequences of MAPK inhibition were further verified on postovulatory oocytes arrested at MII. These oocytes, which had their MAPK activated in vivo, were placed into PD098059 after their recovery from the oviduct and were monitored morphologically after 20 h of incubation. The postovulatory oocytes (Fig. 6C1) were also parthenogenically activated and extruded PBII (Fig. 6C2). The activation of these oocytes was driven further toward female pronucleus formation and early embryonal cell divisions (Fig. 6C, 3 and 4).

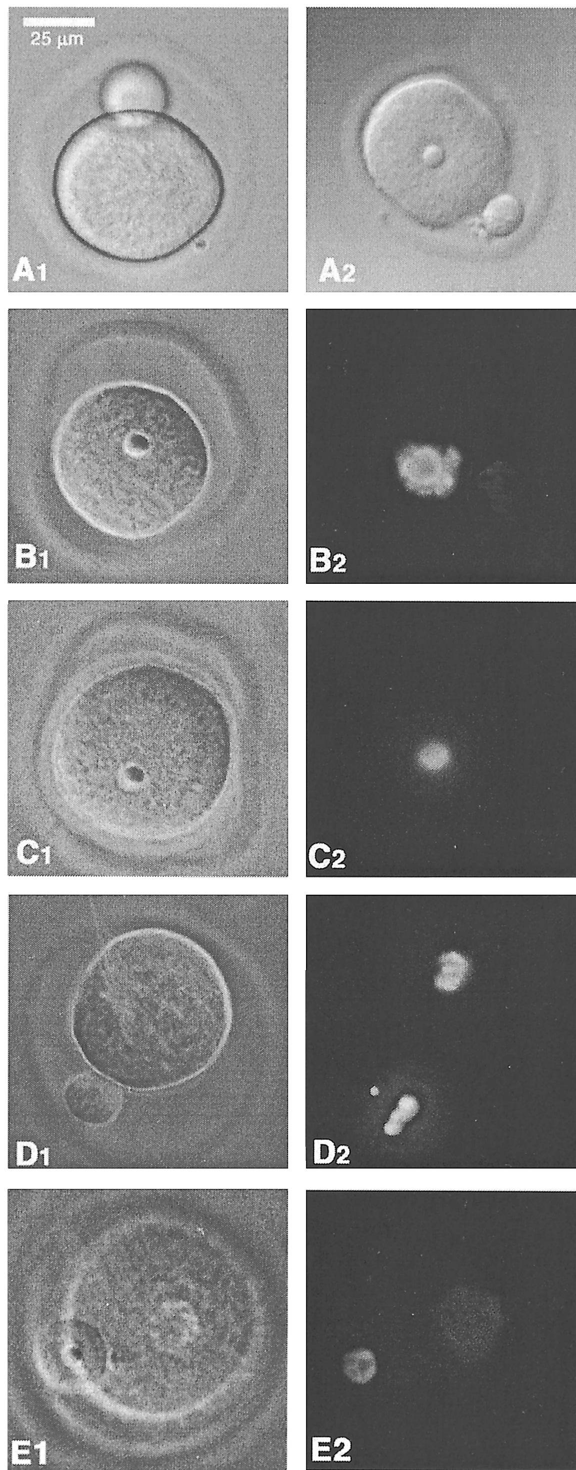

FIG. 5. MPF inhibition results in an artificial interphase. Oocytes maturing spontaneously in vitro were individually selected before or immediately after PBI extrusion and then incubated in 30 μM roscovitine for an additional 12 h. At the end of the incubation period, the oocytes were monitored morphologically by differential interference-contrast microscopy (**A**) and then fixed (**B**–**E**) and had their DNA stained by DAPI and examined by phase-contrast (1) or fluorescent microscopy (2). **A**1) PBI extrusion in control oocytes incubated for 24 h. **A**2) Roscovitine-treated oocyte in which a well-defined nucleus appeared in the oocyte. **B**1) Prophase-arrested oocyte displaying a GV. **B**2) Perinuclear staining of the DNA at the GV. **C**1) An oocyte selected before PBI extrusion and further incubated in 30 μM roscovitine overnight. Note the appearance of a well-defined nucleus. **C**2) Partially condensed chromatin, which has not yet formed chromosomes within the nucleus. **D**1) Extrusion of PBI in a control oocyte incubated in inhibitor-free medium for 24 h. **D**2) An overlay of two images of this oocyte taken at different focal planes. Note segregation of chromosomes between the oocyte and PBI. **E**1) An oocyte selected after PBI extrusion and further incubated in 30 μM roscovitine overnight. Note the appearance of a well-defined nucleus within the oocyte and PBI. **E**2) An overlay of two images of this oocyte taken at different focal planes. Both nuclei contain decondensed chromatin.

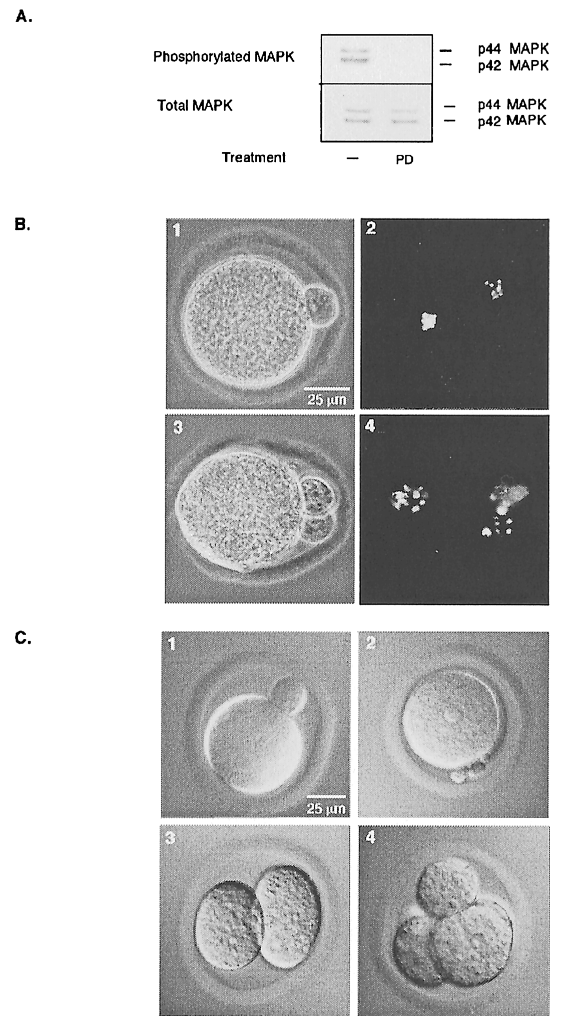

FIG. 6. Prevention of MAPK activation results in parthenogenic activation. **A**) Postovulatory oocytes were placed into either PD098059-containing medium (50 μM) or inhibitor-free medium for an overnight incubation. The oocytes were extracted and subjected to SDS-PAGE, followed by immunoblotting against phosphorylated and total MAPK. A representative result of at least three individual experiments is presented. **B**) Prophase-arrested oocytes were placed into either PD098059-containing medium (50 μM) or inhibitor-free medium. Following an overnight incubation, the oocytes were fixed and stained by DAPI and examined by phase-contrast or fluorescent microscopy. 1) Extrusion of the PBI in a control oocyte. 2) Homologous-chromosome segregation between the oocyte and PBI in an overlay of two images taken at different focal planes. 3) A parthenogenically activated, PD098059-treated oocyte that extruded PBII. 4) Three sets of condensed chromatin, one within the ooplasm and two within each polar body. **C**) Postovulatory oocytes were placed into either PD098059-containing medium (50 μM) or inhibitor-free medium for an overnight incubation. 1) A control, MII-arrested oocyte exhibiting PBI. 2) An activated oocyte containing two PBs and a female pronucleus. 3) A 2-cell parthenogenic embryo. 4) A 4-cell parthenogenic embryo.

TABLE 1. Parthenogenic activation in absence of an active MAPK.[a]

	n	GVB oocytes (%)	PBI oocytes (%)	PBII oocytes (%)
Control	256	94.2 ± 3.8	26.8 ± 5.6	4.0 ± 1.5
PD098059	356	91.9 ± 2.2	28.3 ± 7.2	23.7 ± 2.1

[a] Prophase-arrested oocytes were placed into either PD098059-containing medium (50 μM) or inhibitor-free control medium. Following an overnight incubation, the oocytes were monitored morphologically by differential-interference contrast microscopy. Percentage of oocytes displaying GVB, PBI, or PBII out of total oocytes is presented.

DISCUSSION

The interplay between the two major regulatory enzymes of meiosis in mammalian oocytes, MPF and MAPK, has been a subject of several studies during the past 10 years. However, the information generated in this regard is contradictory and, therefore, confusing. Using a rat animal model, we present herein the first, to our knowledge, demonstration in a mammalian system for an MPF-dependent regulatory step of MAPK activation at reinitiation of meiosis that involves the expression of Mos. We also suggest that the upstream regulator of this cascade is cAMP. Additionally, evidence is provided that once MI has ended, MPF reactivation secures the oocyte from entering interphase.

Interaction of the two pivotal regulatory enzymes, MPF and MAPK, during the meiotic cell cycle has been studied most extensively in amphibian oocytes. Some of these studies show that MAPK controls MPF activity, whereas other reports demonstrate that the converse situation is also true (reviewed in [7, 10, 11]). Activation of MAPK that is regulated by MPF was demonstrated in the pioneer studies that purified MAPK from *Xenopus* oocytes [42, 43]. Additional studies showed that dominant negative p34cdc2 mutants, inactivating p34cdc2 antibodies and the p21cip1 cyclin-dependent kinase inhibitor, all blocked MAPK activation and Mos expression [15, 16, 44]. A reversed hierarchy, however, is suggested by demonstrations that microinjection of mos into *Xenopus* oocytes induced MPF activation [45, 46], further promoting oocyte maturation [12, 47]. Along this line of evidence, antisense oligonucleotide against mos [12, 13] as well as neutralizing antibodies against MEK [14] prevented the activation of MPF at reinitiation of meiosis. In disagreement with the above, an MAPK-independent MPF activation has been suggested by a recent study showing that abrogation of MAPK activity in *Xenopus* oocytes did not affect MPF activation on resumption of meiosis [18].

In mammals, the possible role of MAPK in MPF activation was ruled out by the observation that oocytes derived from mos knockout mice, which are unable to activate MAPK, display a normal pattern of MPF activation [24–26]. Furthermore, the temporal relationship of MPF and MAPK kinetics of activation in rodent oocytes [23, 34, 39] suggests that MPF might be an upstream regulator of the MAPK/Mos pathway. Surprisingly, this option has never been pursued in mammalian oocytes. Indeed, we prove herein that MAPK activation was virtually eliminated in the absence of MPF. Specificity of the MPF inhibitor was conferred by its inability to interfere with the activity of immunoprecipitated MAPK in vitro. Furthermore, employment of a functional phosphorylation assay, in addition to detection of the active from of the enzyme by Western blot analysis, suggests that it is the activation rather than the activity of MAPK that is affected by roscovitine. The inhibition of MAPK activation is correlated with the obstructive effect of roscovitine on accumulation of Mos, the upstream regulator of MAPK. Taken together, the absence of MAPK activation under inhibition of p34cdc2 via repression of Mos expression suggests a linear relationship between these three enzymes. Another study in our laboratory has demonstrated recently that the expression of Mos is subjected to negative regulation by a protein kinase A-mediated cAMP action [39]. As cAMP represses MPF activity, the linear relationship between MPF, Mos, and MAPK can be extended to include cAMP as their upstream negative regulator.

As expected, we show that interference with the catalytic activity of p34cdc2 at the onset of meiosis prevented MPF activation and blocked the oocyte at the GV stage [37, 48]. More interesting is our additional observation that inhibition of MPF activity just before polar body extrusion prevented the exit from MI. Under these conditions, the oocytes had a ‘‘reversed’’ phenotype, in which a nucleus had reformed. These findings suggest that the maximal MPF activity observed at MI is of major significance in regulating meiosis progression. This original observation reveals that in addition to the initial role of MPF at the onset of meiosis, its sustained activity is absolutely necessary to ensure meiotic progress.

The enzyme responsible for suppression of DNA replication at the exit from MI has been a subject of dispute in several studies. Formerly, it has been postulated that the sustained MAPK activity in between the two meiotic divisions prevents DNA replication. This notion was fortified by experimental evidence showing that in *Xenopus* oocytes, the mere ablation of Mos (either by using the antisense strategy or by the microinjection of antibodies) results in the formation of a nucleus and replication of DNA [17, 49]. Early experiments in mouse oocytes utilizing the same techniques seem to indicate that, without Mos, the nucleus is reformed and the DNA replicates, similar to *Xenopus* oocytes [19, 20, 21, 27, 50]. However, use of the Mos knockout mice, generated more recently, disclosed that contrary to the above reports, interkinesis occurs in the absence of an active MAPK [24, 29, 30].

Using an MEK inhibitor, we also show herein that in the rat, interkinesis occurs normally in the absence of an active MAPK. However, our study not only demonstrates that MAPK is not responsible for the prevention of interphase but also provides evidence that MPF reactivation is absolutely necessary for maintenance of interkinesis. This evidence is provided here by inhibition of MPF at the onset of MII that misleads the oocyte to a transition into interphase, as manifested by the presence of the well-defined nucleus and the decondensed chromatin. To our knowledge, the present study represents the first demonstration of the requirement of an active MPF for the suppression of interphase. These findings are in accordance with previous reports in starfish and mouse oocytes showing that p34cdc2 repression during MI brought about DNA replication between the two meiotic divisions [51, 52]. We further demonstrate that the artificial interphase induced by the deficiency in MPF activity occurs in the presence of an active MAPK. Hence, MAPK is not involved in interphase suppression.

The role of the Mos/MAPK pathway in mammalian oocytes was deciphered in the mos knockout mice [29, 30]. Oocytes derived from these mutant mice are as expected, unable to activate MAPK, but they are able to proceed normally through early meiotic events, displaying a normal

pattern of MPF activation [24–26]. The only default of these oocytes is their inability to arrest at MII; they undergo parthenogenic activation in the absence of sperm. The requirement for an active MAPK for second metaphase arrest is also demonstrated in our study. We show herein that similar to Mos deficiency, pharmacological inhibition of MAPK did not prevent the oocyte from completing the first round of meiosis and emitting PBI but did impair the ability to arrest at MII. These oocytes were parthenogenically activated, forming PBII without fertilization. In some oocytes, this activation was carried further to pronucleus formation and even proceeded through the first and second mitotic divisions, forming 2- and 4-cell embryos. The mechanism involved in MAPK-regulated MII arrest in rodent oocytes has not been investigated. Nonetheless, the mediatory role of p90rsk that has been demonstrated in *Xenopus* [18] can possibly apply to mammals as well.

Quite interestingly, inhibition of MPF reactivation was accompanied by rephosphorylation of p34cdc2. Previously, we showed rephosphorylation of p34cdc2 in 1-cell rat embryos exiting metaphase II upon fertilization [53]. The appearance of rephosphorylated p34cdc2 under conditions of its inhibition may represent a regulatory loop that exists between the enzyme and its regulators. Specifically, in the absence of an active p34cdc2, its rephosphorylation may be mediated by either the lack of an active cdc25, the phosphatase that converts the pre-MPF into an active kinase, or by the presence of an active Wee1 that phosphorylates p34cdc2 [54]. Indeed, cdc25 has been reported to be activated by p34cdc2 [40]. Furthermore, Wee1 in *Xenopus* has been shown to be hyperphosphorylated and inactivated by p34cdc2 [54]. In the same line of evidence, overexpression of Wee1 in *Xenopus* meiotic extract resulted in the rephosphorylation of p34cdc2 and entry into interphase [55]. Expression of both Wee1 and cdc25 have been shown in mouse oocytes [56]; however, the activity of these enzymes in mammals has not been studied as yet. Rephosphorylation of p34cdc2 under conditions of low MPF activity demonstrated herein in the rat can provide a preliminary clue for the presence of a regulatory loop between p34cdc2 and Cdc25 and/or Wee1 in the mammalian oocyte.

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