

Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes

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SUMMARY

Oocyte meiotic maturation is triggered by different stimuli (hormones, unknown signals through cell interactions) in different species. These stimuli indirectly lead to the activation of a major cell cycle regulating activity, the maturation promoting factor (MPF). Other factors, such as the product of the proto-oncogene *c-mos* or enzymes of the MAP kinase family, are also involved in the process of maturation. MAP kinase activation occurs during meiotic maturation in oocytes from different species with different kinetics. The relationships between MPF activation and MAP kinase activation have been well studied in species such as clam and *Xenopus*. In this paper, we study the precise timing of MAP kinase activation (as measured by phosphorylation of exogenous myelin basic protein and shifts in mobility of ERK 1 and ERK 2) versus MPF activation (as measured by phosphorylation of exogenous histone H1) during mouse oocyte maturation and, in parallel, morphological events such as changes in microtubule organization and chromatin condensation. We observed that MAP kinase activation was delayed after

MPF activation and that this activity persisted throughout maturation whereas MPF activity dropped between the two meiotic metaphases. After parthenogenetic activation of ovulated eggs, MAP kinase inactivation was very slow compared to MPF inactivation. During the first mitotic cell cycle, a rise in myelin basic protein kinase activity at M-phase was observed but it was not related to MAP kinase activation. Furthermore, microtubules and chromatin remained in a metaphase-like state during the complete period of maturation (including the period between the two meiotic metaphases) and a few hours after activation. Thus, during meiosis but not during mitosis, the changes in microtubule organization and chromatin condensation correlate with MAP kinase activity rather than with MPF activity. We discuss the possible role of MAP kinase in the maintenance of a metaphasic state during meiosis when MPF is inactive.

Key words: mouse oocytes, MAP kinase, H1 kinase, microtubules, meiosis, mitosis

INTRODUCTION

Fully grown mouse oocytes are arrested at the G₂/M border of the first meiotic division. The removal of these oocytes from the follicles induces the release from this arrest, characterized by the breakdown of the germinal vesicle (GVBD) and the resumption of meiotic maturation. A second arrest occurs at metaphase of the second meiotic division, which is released only by fertilization or parthenogenetic activation and is followed by further embryonic development. The cell cycle during maturation is controlled by a major factor called maturation promoting factor (MPF). MPF is composed of a catalytic subunit, p34^{cdc2} possessing an histone H1 kinase activity and a regulatory subunit, a cyclin. This factor is activated at GVBD and its activation triggers chromatin condensation, nuclear envelope breakdown and the formation of the first meiotic spindle. The cell cycle transition from interphase to metaphase is accompanied by extensive rearrangement of the microtubule

network. Interphase microtubules are long and relatively stable, and radiate throughout the cytoplasm, while metaphase microtubules are short, dynamic and restricted to the region of the spindle. MPF can induce the reorganization of interphase microtubules toward a metaphase configuration in vitro (Verde et al., 1992).

The cell cycle of oocytes undergoing meiosis, however, differs from a typical mitotic cell cycle (Kubiak et al., 1992). The formation of the first meiotic spindle is slow and takes about 6 hours whereas the formation of the second meiotic spindle takes only about 1 hour. During the transition between metaphase I and metaphase II and for several hours after activation, both the chromosomes and the microtubules remain in a metaphase-like configuration, despite a low histone H1 kinase activity (Kubiak et al., 1992; Szöllösi et al., 1993). Thus, the organization of microtubules and chromosomes during meiotic maturation does not follow precisely changes in MPF activity. In addition, there is an increase in phosphate

incorporation into proteins during the metaphase I to metaphase II transition in mouse oocytes (Kubiak et al., 1992), while normally the level of protein phosphorylation is high during M-phase. This observation suggests that kinases other than MPF are active during the metaphase I-metaphase II transition.

The mitogen-activated protein (MAP) kinase, also named extracellular regulated kinase (ERK), is a serine/threonine kinase that is activated by phosphorylation of specific threonine and tyrosine residues (Cobb et al., 1991; Payne et al., 1991; Seger et al., 1991; Posada and Cooper, 1992). Genes encoding MAP kinase have been cloned in different species (Boulton et al., 1990) and related kinases, *FUS3* (Elion et al., 1990), *KSS1* (Courchesne et al., 1989) and *MPK1* (Lee et al., 1993), have been characterized in the budding yeast. MAP kinase lies on the signalling pathway by which an external stimulus at the cell surface causes a cell cycle event. Genetical and biochemical studies have unravelled part of the signalling pathway leading to MAP kinase activation that appears to be conserved from yeast to vertebrates (for review see Errede and Levin, 1993; Ruderman, 1993). The kinase responsible for MAP kinase phosphorylation, the MAP kinase activator (MAPKK), is also a protein kinase activated by phosphorylation on threonine (Matsuda et al., 1992; Nakielny et al., 1992; Seger et al., 1992). A transient activation of MAP kinase occurs after quiescent, G₀-arrested tissue culture cells are serum-stimulated and reenter the cell cycle (for review see Cobb et al., 1991).

MAP kinase is also stimulated during meiotic maturation in clam (Shibuya et al., 1992), *Xenopus* (Ferrel et al., 1991; Gotoh et al., 1991b; Posada et al., 1991), mouse oocytes (Verlhac et al., 1993), and thus probably in other species. Clam oocyte maturation is triggered by fertilization and displays a transient activation of MAP kinase that begins before GVBD and ends at anaphase of the first meiotic division (Shibuya et al., 1992). In *Xenopus* oocytes however, MAP kinase activation occurs at GVBD, concomitantly with MPF activation, and its activity persists during the whole process of maturation (Ferrel et al., 1991; Gotoh et al., 1991b; Posada et al., 1991). MPF triggers MAP kinase activation when microinjected into *Xenopus* immature oocytes, whereas MAP kinase is not able to induce MPF activation using the same procedure (Gotoh et al., 1991a). MAP kinase is also involved in the regulation of microtubule dynamics in *Xenopus* eggs extracts in vitro (Gotoh et al., 1991b). Mouse oocytes possess at least two forms of MAP kinase, p42-ERK 2 and p44-ERK 1 that become phosphorylated during meiotic maturation as shown by their mobility on SDS-PAGE, where the phosphorylated forms migrate slower than the non-phosphorylated ones (Verlhac et al., 1993). In this study, we performed a detailed analysis of mouse meiotic maturation assessing in parallel the phosphorylation state of ERK 1 and ERK 2, the kinetics of MAP kinase and histone H1 kinase activity, the distribution of the microtubule network and the state of condensation of chromatin.

MATERIALS AND METHODS

Collection and culture of oocytes

To obtain immature oocytes arrested at prophase I of meiosis, 5- to 6-week-old Swiss female mice (Animalerie Spécialisée de Villejuif,

Centre National de la Recherche Scientifique, France) were killed and their ovaries were removed and transferred to warmed (37°C) M2 medium (Whittingham, 1971) supplemented with 50 µg/ml dibutyryl cyclic AMP (dbcAMP). The ovarian follicles were punctured to release the enclosed oocytes and immature oocytes displaying a germinal vesicle (GV) were collected. The dbcAMP prevents the immature oocytes from undergoing germinal vesicle break down (GVBD). After a careful wash, immature oocytes were cultured in M2 medium under liquid paraffin oil at 37°C.

To obtain ovulated eggs, 5- to 6-week-old Swiss female mice were superovulated by injection of 5 i.u. of pregnant mare serum gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet), 48 hours apart. Mice were killed 15 hours post-hCG, the egg masses were released from the oviducts and the cumulus cells surrounding the eggs were removed by a brief exposure to 300 i.u./ml hyaluronidase (Boehringer) in M2 medium. After a series of washes, the eggs were cultured in M2 medium as described above.

Parthenogenetic activation of ovulated eggs

Ovulated oocytes were activated according to the method of Cuthbertson (1983) by a 6.5 minutes exposure to freshly prepared 8% ethanol (Merck) in M2 medium. After extensive washing oocytes were cultured in M2 medium.

Immunofluorescence

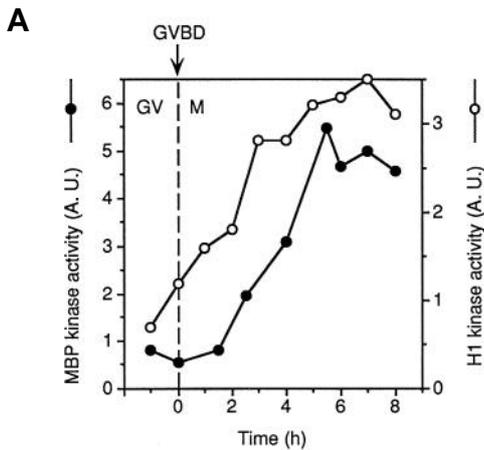
The oocyte fixation and labeling were performed as described before (Kubiak et al., 1992). We used the rat monoclonal antibody YL1/2 specific for tyrosinated α -tubulin and a fluorescein-conjugated anti-rat (Miles) secondary antibody. The chromatin was visualized using propidium iodide (Molecular Probes; 5 µg/ml in PBS). Samples were observed with a Bio-Rad MRC-600 confocal microscope.

Immunoblotting

100 oocytes at the appropriate stage of maturation were collected in sample buffer (Laemmli, 1970) and heated to 100°C for 3 minutes. The proteins were separated by electrophoresis in 10% polyacrylamide (ratio acrylamide/bis-acrylamide: 100/1) containing 0.1% SDS and electrically transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0.45 µm). Following transfer and blocking for 2 hours in 3% skimmed milk in 10 mM Tris (pH 7.5), 140 mM NaCl (TBS), containing 0.1% Tween-20, the membrane was incubated overnight at 4°C with the primary antibody (anti-ERK, n° 691 Santa Cruz Biotechnology) diluted 1:200 in blocking solution. After three washes of 10 minutes each in 0.1% Tween-20/TBS, the membrane was incubated for 1 hour at room temperature with an anti-rabbit antibody conjugated to horseradish peroxidase (Amersham) diluted 1:300 in 3% skimmed milk in 0.1% Tween-20/TBS. The membrane was washed three times in TBS/Tween and then processed using the ECL detection system (Amersham). All experiments were repeated at least twice.

Kinase activity assays

The histone H1 kinase activity was measured as previously described (Kubiak et al., 1992). The H1 kinase activity in whole lysate is the sum of all H1 kinases in the cell, including many cyclin-dependent kinases. For this reason, 10 µM inhibitor of cAMP-dependent protein kinase (Sigma) was added in the assay. However, we did not find any difference in histone H1 kinase activity measured with or without the inhibitor. The myelin basic protein (MBP) kinase activity was determined following Boulton et al. (1991). 30 oocytes were transferred into 4 µl of M2 medium, frozen immediately in a mixture of dry ice and ethanol, and stored at -80°C. For the kinase assays, 2 µl of reaction buffer (4×, 100 mM HEPES-NaOH, pH 8, 40 mM MgCl₂, 4 mM DTT, 4 mM benzamidine, 120 µM ATP, 20 mM NaF, 0.4 mM sodium orthovanadate) supplemented with protease inhibitors was added to the oocyte sample, which was then thawed and refrozen twice to lyse the cells. The reaction was started by addition of 2 µl of



a solution containing 1.5 mg/ml MBP, 4 mM ATP, and 0.5 mCi/ml γ -[32 P]ATP and lasted 30 minutes at 30°C. The reaction was stopped by adding 2 \times sample buffer (Laemmli, 1970) and boiling for 3 minutes. The samples were then analysed by electrophoresis in 15% polyacrylamide gels containing 0.1% SDS followed by autoradiography.

Some MBP kinase assays were performed in 10% polyacrylamide gels containing 0.5 mg/ml MBP following SDS-PAGE as described in Shibuya et al. (1992) using samples of 200 oocytes, at appropriate stages of development.

All experiments were repeated at least three times.

RESULTS

MAP kinase is activated after MPF activation in oocytes undergoing meiotic maturation

By scoring germinal vesicle breakdown, we synchronized a population of oocytes undergoing meiosis and were therefore able to follow precisely their progression into the meiotic cell cycle. To determine the kinetics of activation of MAP kinase and MPF (Fig. 1A), we collected samples of maturing oocytes and assayed them for their capacity to phosphorylate MBP (MAP kinase) and histone H1 (cdc2 kinase). H1 kinase activity doubled at GVBD as compared to the GV stage (Fig. 1A). In contrast MBP kinase activity did not increase until 2 to 3 hours

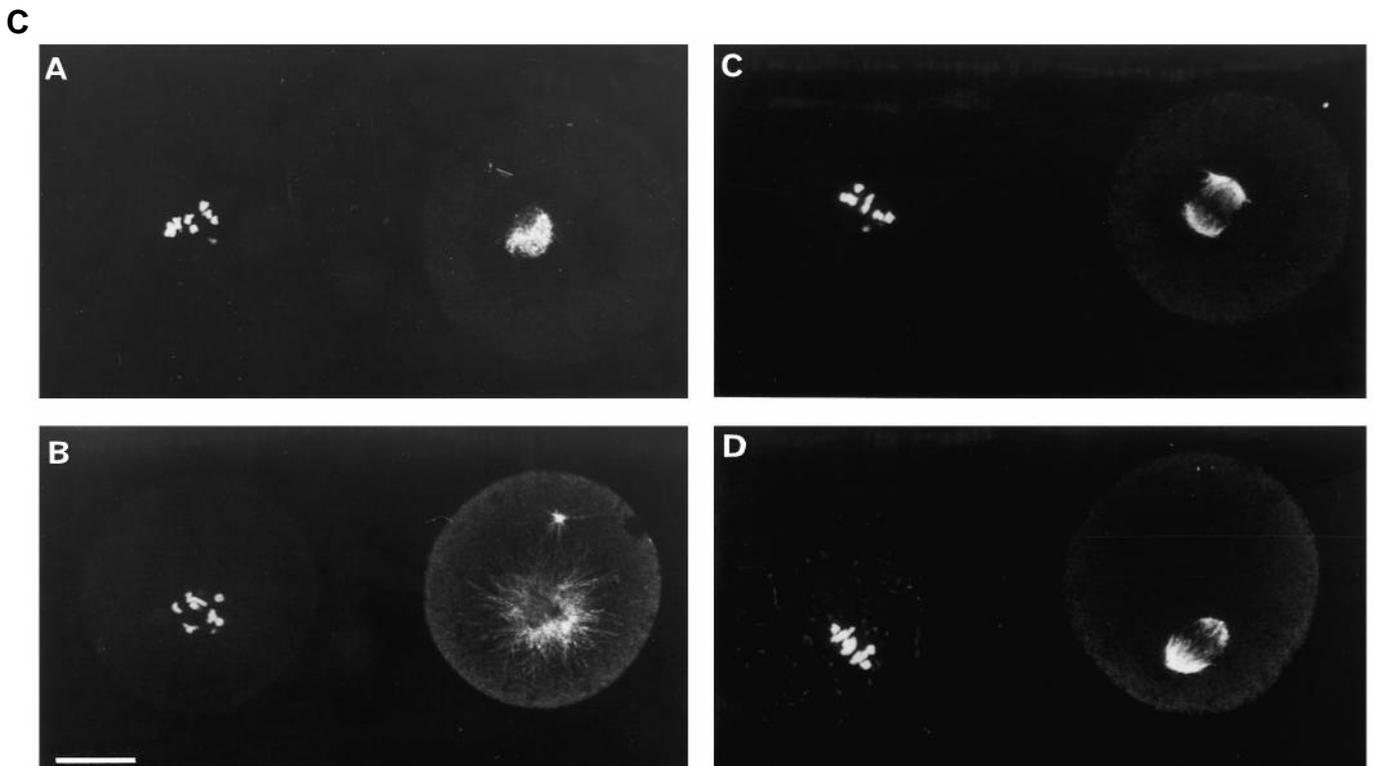
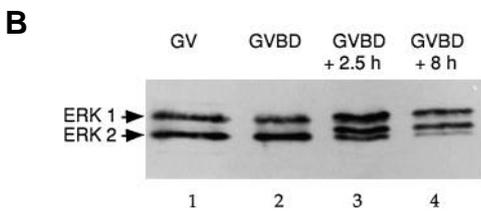


Fig. 1. Resumption of meiotic maturation is followed by a delay in MAP kinase activation compared to MPF activation, and a slow spindle formation. (A) Kinetics of histone H1 and MBP kinase activities during metaphase I of meiotic division. GVBD was considered as t=0. (B) Lysates of GV-stage oocytes or maturing oocytes were analyzed by immunoblotting using an anti-ERK antiserum. Lane 1, GV-stage oocyte; lane 2, oocytes taken at GVBD; lane 3 and 4, oocytes collected at 2.5 and 8 hours after GVBD respectively; GV, germinal vesicle; GVBD, germinal vesicle breakdown; M, M-phase. (C) Maturing oocytes were fixed at different times after GVBD and double stained with propidium iodide for the chromosomes, and with fluoresceine for the microtubules. For all panels, chromosomes are on the left and microtubules are on the right. (A-D) Oocytes were fixed 1 hour, 1.5, 3 and 5 hours after GVBD respectively. Scale bar, 25 μ m.

after GVBD. Both the MBP kinase and histone H1 kinase activities reached their maximum 5 to 6 hours after GVBD. In parallel, we analysed mobility shifts on immunoblots to assess the phosphorylation state of ERK 2 and ERK 1 (Fig. 1B). Oocytes taken at the GV stage (Fig. 1B lane 1) or at GVBD (Fig. 1B lane 2) displayed two bands of relative molecular mass (M_r) 42 and 44×10^3 . Oocytes collected at 2.5 hours after GVBD displayed both the fast migrating and the slow migrating forms of ERK 1 and ERK 2 (Fig. 1B lane 3). By 8 hours after GVBD, almost all ERK 2 and ERK 1 proteins had shifted to their slow migrating forms, of respectively M_r 44 and 46×10^3 (Fig. 1B lane 4). Thus, MAP kinase activation was delayed by several hours compared to MPF activation, as assayed by phosphorylation of MBP *in vitro* and mobility shifts.

We analysed at the same time changes in microtubule distribution by immunofluorescence (Fig. 1C). A high background and no microtubules were visible at the time of germinal vesicle breakdown (data not shown). 30 minutes after GVBD, short microtubules were present in the vicinity of the condensing chromosomes (panel A). This microtubular organization remained until 1.5 hours after GVBD. At this time, long microtubules radiated from the chromosomes and cytoplasmic asters were also sometimes visible (panel B). This pattern was transitory and, by 2 hours after GVBD, these long microtubules shortened and the chromatin was more condensed (data not shown). By 3 hours after GVBD, we could see first bipolar microtubular structures with some chromosomes aligned on the metaphase plate and others to the sides (panel C). By 5 hours after GVBD, a clearly defined spindle, more furnished in microtubules, and with chromosomes aligned on the metaphase plate, was visible (panel D). The assembly of the first meiotic spindle correlated better temporally with MAP kinase activation than with MPF activation.

During the transition between the two meiotic metaphases MPF activity drops while MAP kinase activity stays high

ERK 1 and ERK 2 remain phosphorylated during the metaphase I to metaphase II transition (Verlhac et al., 1993) while MPF activity drops (Kubiak et al., 1992). Ethanol treatment of freshly ovulated metaphase II (MII) oocytes triggers the extrusion of the second polar body and the entry into a subsequent metaphase, called metaphase III (MIII; Kubiak, 1989). After extrusion of the second polar body, the metaphase III oocyte is arrested in M-phase and possesses a spindle with a haploid content of chromosomes with single chromatids. This abortive activation leading to MIII, mimics the natural metaphase I/metaphase II (MI/MII) transition in terms of both morphological and biochemical events (Kubiak et al., 1992). Furthermore, it is a convenient system that provides large numbers of synchronized oocytes and enabled us to examine simultaneously the histone H1 kinase activity, the MBP kinase activity, the MAP kinase phosphorylation state and the morphological state of the chromosomes and of the microtubules.

During the MII/MIII transition, the histone H1 kinase activity dropped four-fold at the time of polar body extrusion (Fig. 2A). This activity increased again in MIII oocytes, to a level slightly lower than in MII oocytes. In contrast, the MBP kinase activity remained high during this transition (Fig. 2A).

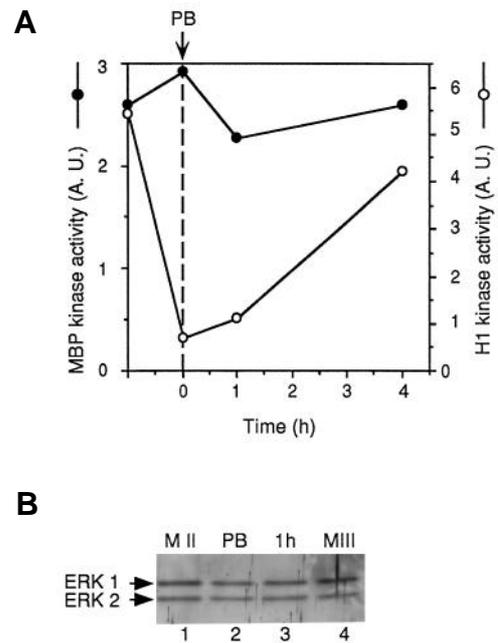


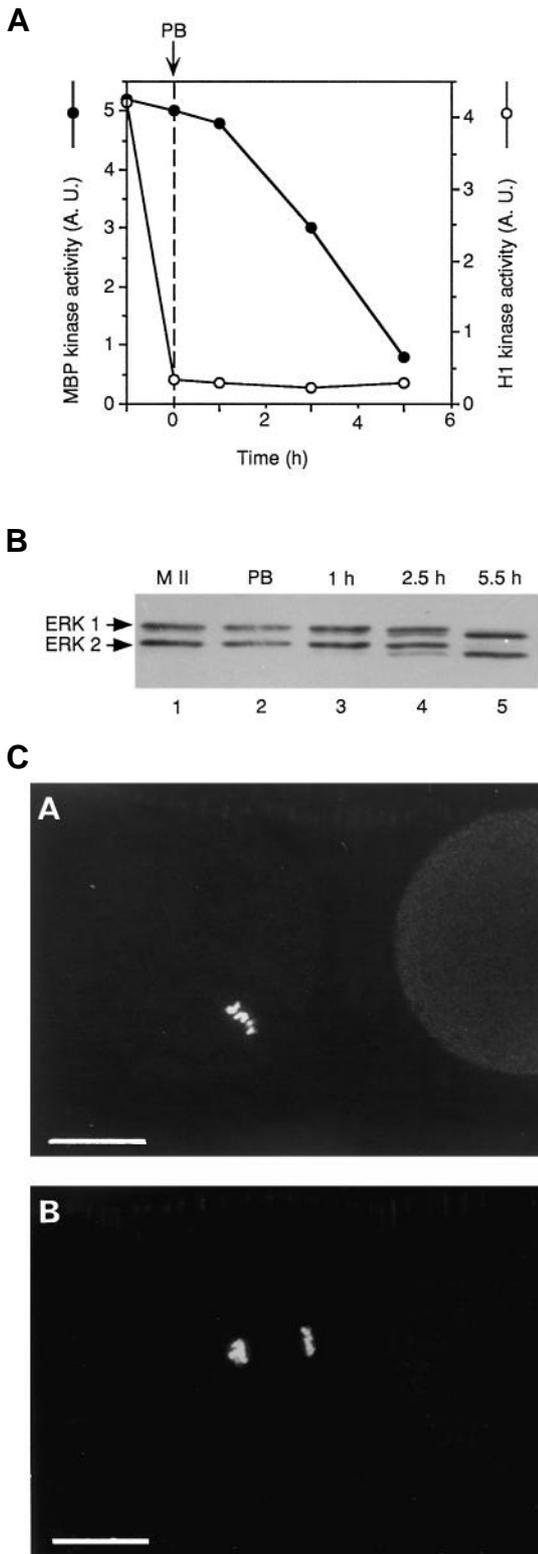
Fig. 2. MAP kinase activity remains high during the metaphase II to metaphase III transition while MPF activity drops. (A) Kinetics of histone H1 and MBP kinase activities during the MII/MIII transition. (B) Lysates of MII-arrested oocytes, or oocytes collected after early activation, were analyzed by immunoblotting using an anti-ERK antiserum. Lane 1, MII-arrested oocytes; lane 2, oocytes collected at second polar body extrusion; lane 3, oocytes collected 1 hour after polar body extrusion; lane 4, MIII-arrested oocytes; MII, metaphase II; MIII, metaphase III. The MIII oocytes were collected 3 hours after PBE, in order to discriminate them from the pronucleated oocytes; PB, polar body.

Similarly, the M_r of ERK 1 and ERK 2 was unchanged and identical to the high M_r present in metaphase II oocytes (Fig. 2B lane 1 to 4). The chromosomes did not decondense and the microtubules remained 'metaphasic' (data not shown) during this transition as observed previously (Kubiak et al., 1992). Therefore changes in microtubular organization correlated with MAP kinase activity rather than with MPF activity during the MII/MIII transition.

After parthenogenetic activation of ovulated eggs, the inactivation of MAP kinase is very slow compared to H1 kinase inactivation

Parthenogenetic activation, in contrast to fertilization, leads to a large population of synchronized eggs entering interphase. The beginning of the second polar body extrusion takes place 45 minutes after ethanol activation and pronuclei are clearly visible 4 hours later in the 1-cell embryo.

We observed a dramatic drop of histone H1 kinase activity during extrusion of the second polar body (Fig. 3A). In contrast, the MBP kinase activity remained high even 1 hour after polar body extrusion (PBE). By 3 hours after PBE, the MBP kinase activity was reduced by 50% and it declined to basal level 5 hours after PBE. This slow decrease in MBP kinase activity correlated precisely with the slow dephosphorylation of MAP kinase: ovulated eggs (Fig. 3B lane 1) as well as eggs collected at PBE (Fig. 3B lane 2), or 1 hour after PBE (lane 3) displayed the slow migrating forms of ERK 1 and ERK



2. The fast migrating forms were first detected in eggs collected 2.5 hours after PBE (lane 4). By 5 hours after polar body extrusion, only the fast migrating forms of ERK 1 and ERK 2 were observed (lane 5). Thus, dephosphorylation of MAP kinase and loss of MBP kinase activity did not occur until several hours after loss in MPF activity.

We analysed at the same time the microtubular changes occurring during the metaphase II to interphase transition (Fig. 3C). Metaphase II-arrested oocytes contained a barrel-shaped spindle positioned near the cortex (Fig. 3A). After activation, the oocytes expelled their second polar body and a large microtubular structure, the midbody, joined the polar body and the oocyte (Fig. 3B). Except for the midbody, no microtubules were visible in the cytoplasm 30 minutes after polar body extrusion and the chromosomes remained condensed (Fig. 3B). At 1.5 hours after PBE, some asters containing long microtubules appeared, and long microtubules grew in the oocyte near the chromosomes (Fig. 3C). The microtubular network then intensified in the cytoplasm and by 4 hours after PBE it became very dense and rich in long microtubules (Fig. 3D). At this time, the chromatin was decondensed and pronuclei were clearly visible under phase contrast. No interphasic microtubules were observed until the beginning of MAP kinase inactivation.

We have performed a similar experiment after in vitro fer-

Fig. 3. After activation of ovulated oocytes, the slow inactivation of MAP kinase correlates with the changes in microtubules and chromatin organization. (A) Kinetics of histone H1 and MBP kinase activities during the metaphase II to interphase transition. Polar body (PB) extrusion was taken as $t=0$. (B) Lysates of MII-arrested oocytes, or oocytes collected after activation, were analyzed by immunoblotting using an anti-ERK antiserum. Lane 1, MII-arrested oocytes; lane 2, oocytes collected at polar body extrusion; lanes 3, 4 and 5, oocytes collected at 1, 2.5 and 5.5 hours after polar body extrusion respectively. (C) Oocytes, collected at different times during the metaphase II to interphase transition, were fixed in glutaraldehyde. They were then double labeled with propidium iodide to show chromosomes and with fluorescein to show microtubules. On all panels, chromosomes are on the left and microtubules on the right. (A) Oocytes were fixed at the metaphase II stage; (B-D) oocytes were fixed at 0.5 hour, 1.5 hours and 4 hours after polar body extrusion respectively. Scale bar, 25 μ m.

tilization, where the oocytes were exposed to spermatozoa for 40 minutes. Although the population was highly heterogenous and less synchronous (mono-, di-, or tri-spermic eggs at different stages), we observed that the MBP kinase inactivation occurred between 2 and 3 hours after the drop in histone H1 kinase activity, which took place at polar body extrusion. Similarly, a dense microtubular network appeared in the eggs 2 hours after polar body extrusion (data not shown).

MAP kinase is not re-activated during the first mitotic cleavage division

In *Xenopus* oocytes, an MBP kinase activity is detected during metaphase of the first mitotic cell cycle (Gotoh et al., 1991b) although p42^{mapk} does not become phosphorylated at this time (Ferrel et al., 1991). Since MAP kinase activity depends on threonine and tyrosine phosphorylation (Posada and Cooper, 1992), this observation suggested that the mitotic MBP kinase activity was not related to p42^{mapk}.

To determine whether MAP kinase was active at mitotic metaphase in mouse eggs, we activated eggs 18-19 hours post-hCG, then cultured and scored them 12 to 14 hours later for the disappearance of the pronuclear membranes. 70% of the eggs had reached metaphase by 14 hours after activation and the mean duration of the first mitosis was 2 hours. Eggs sampled before the breakdown of the pronuclear membrane or after cytokinesis, at the 2-cell stage, possessed a low MBP kinase activity (Fig. 4A). In contrast, eggs sampled in M phase of the first embryonic mitosis displayed an MBP kinase activity whose level was comparable to the MBP kinase activity of metaphase II-arrested oocytes (Fig. 4A). Despite the elevated MBP kinase activity, only the fast migrating (non-phosphorylated) forms of ERK 1 and ERK 2 were observed, as in interphase eggs (Fig. 4B).

To determine whether the mitotic MBP kinase activity observed *in vitro* was related to ERK proteins, we assayed MAP kinase directly within an SDS gel. In metaphase II-arrested oocytes, two major MBP kinase activities were associated with proteins of M_r 44 and 46×10^3 corresponding to the slow migrating forms of ERK 2 and ERK 1 respectively (Fig. 4C lane 1). Few other proteins of higher M_r possessed a weak MBP kinase activity in these oocytes. This confirms that MBP kinase activity was due mainly to p42 and p44. The 44 and 46×10^3 proteins retained this MBP kinase activity in eggs extruding their second polar body (Fig. 4C lane 2). In contrast, eggs at the pronuclear stage or eggs in first mitosis did not possess an MBP kinase activity detectable by this assay (Fig. 4C lane 3 and 4). Thus, the lack of MBP kinase activity in the gel in mitosis was consistent with the absence of MAP kinase phosphorylation in mitosis.

6-DMAP inhibits directly MAP kinase and accelerates its inactivation in oocytes entering interphase

6-dimethylaminopurine (6-DMAP), a kinase inhibitor (Néant and Guerrier, 1988), accelerates the transition from metaphase to interphase in activated eggs as indicated by two lines of evidence (Szöllösi et al., 1993). First, the drug accelerates dephosphorylation of some highly phosphorylated proteins during the transition to interphase. Second, the formation of the interphase network of microtubules and the decondensation of chromatin occur more rapidly (1 hour earlier) in activated

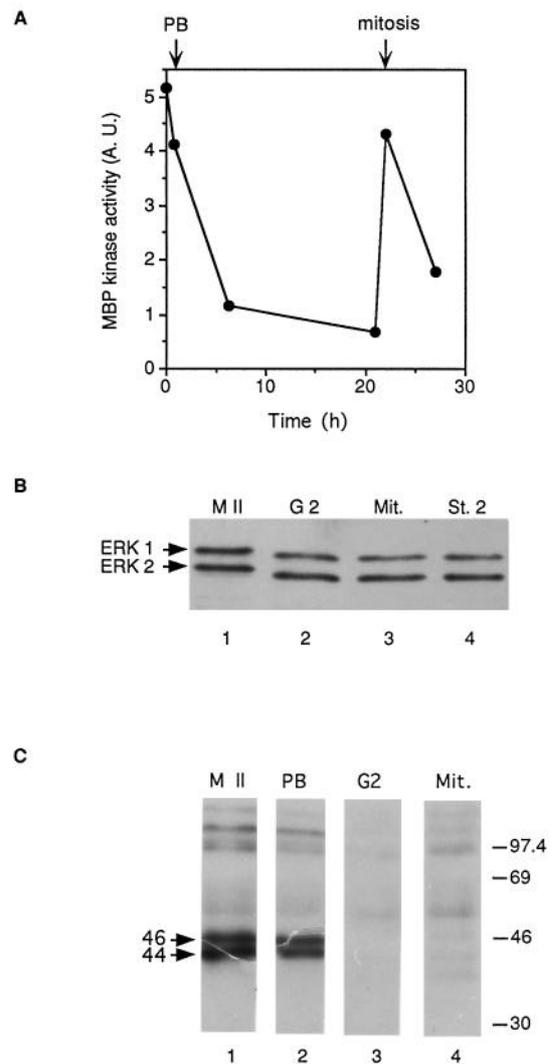


Fig. 4. MAP kinase is not re-activated during the first embryonic mitosis. (A) Changes in MBP kinase activity after activation of ovulated eggs and during mitosis. (B) Lysates of MII-arrested oocytes, or oocytes collected at different times during mitosis, were analyzed by immunoblotting using an anti-ERK antiserum. Lane 1, MII-arrested oocytes; lane 2, oocytes collected just before mitosis and exhibiting pronuclei; lane 3, oocytes collected in M-phase; lane 4, oocytes collected at the 2-cell stage, after mitosis. (C) Lysates of oocytes collected in metaphase II or during the first mitosis were subjected to an in gel MBP kinase assay. Lane 1, MII-arrested oocytes; lane 2, oocytes taken immediately following polar body extrusion; lane 3, oocytes taken just before mitosis; lane 4, oocytes collected in M-phase. PB, polar body; G2, G₂ phase of the cell-cycle; Mit., first embryonic mitosis; St.2, 2-cell stage. Molecular weight markers ($\times 10^{-3}$) are on the right.

eggs treated with 6-DMAP as compared with control activated eggs (Szöllösi et al., 1993). To investigate whether 6-DMAP affected MAP kinase activity, eggs were activated parthenogenetically in the presence of 2.5 mM 6-DMAP, cultured for 1.5 or 2.5 hours, then assayed for MBP kinase activity *in vivo*. When 6-DMAP was present in the *in vitro* assay, it strongly inhibited the MBP kinase (Mean \pm SD: 80% \pm 10%, $n=3$), demonstrating that 6-DMAP inhibits directly MAP kinase activity.

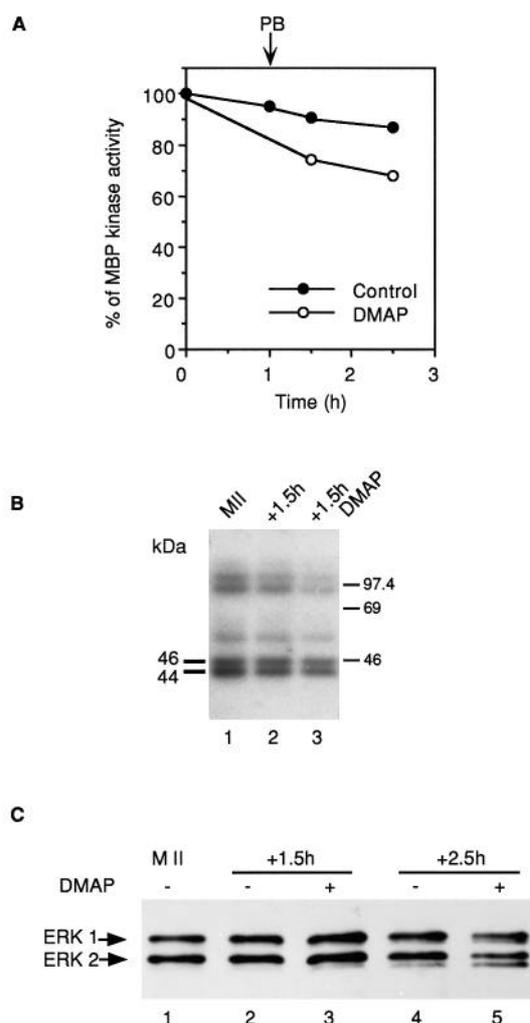


Fig. 5. The kinase inhibitor 6-DMAP induces a more rapid inactivation of MAP kinase during the metaphase II to interphase transition. Ovulated oocytes were activated and cultured in the absence or the presence of 2.5 mM 6-DMAP for 1.5 or 2.5 hours. (A) MBP kinase activity measured at different times during the MII/interphase transition in the presence or absence of DMAP. The percentage of MBP kinase activity was calculated compared to MII-arrested oocytes. The beginning of incubation in DMAP was considered as $t=0$. (B) Lysates of MII-arrested oocytes, or oocytes incubated for 1.5 hours after activation in control medium or in DMAP, were subjected to an in gel MBP kinase assay. Lane 1, 200 MII-arrested oocytes; lane 2, 200 oocytes incubated for 1.5 hours in control medium; lane 3, 200 oocytes incubated for 1.5 hours in DMAP. Coomassie blue staining showed that all lanes contained the same amount of proteins. Molecular weight markers ($\times 10^{-3}$) are on the right. (C) Lysates of MII-arrested oocytes, or oocytes incubated for 1.5 or 2.5 hours, after activation, in control medium or in DMAP, were analyzed by immunoblotting using an anti-ERK antiserum. Lane 1, MII-arrested oocytes; lanes 2 and 3, oocytes incubated for 1.5 hours in control medium, or in DMAP; lanes 4 and 5, oocytes incubated for 2.5 hours in control medium, or in DMAP.

In addition, we studied the effect of the inhibitor on MAP kinase inactivation, since it depends upon its phosphorylation state. Therefore, we washed carefully the eggs treated with 6-DMAP. The MBP kinase activity in eggs activated in the presence of 6-DMAP was slightly reduced (mean: 25% inhi-

bition) in comparison with the control (Fig. 5A). A small drop (22%) in MBP kinase activity associated with proteins of 44 and 46×10^3 was observed in the in gel assay (Fig. 5B). We analysed in parallel the drop in MBP kinase activity and the shift in mobility of ERK 1 and ERK 2 in control eggs and eggs treated with 6-DMAP. Although the MBP kinase activity of eggs incubated in 6-DMAP for 1.5 hours after activation was reduced by 30% as compared to control eggs, we could not detect any shift in mobility associated with this small drop in activity (Fig. 5C lane 3, compare to lanes 1 and 2). However, eggs incubated 1 hour longer in 6-DMAP (eggs sampled at 2.5 hours after activation) showed a drop of 42% in MBP kinase activity, and in these eggs the shift in mobility was more pronounced than in controls (Fig. 5C compare lanes 4 and 5). Eggs treated with 6-DMAP had formed an interphasic microtubule network and contained decondensed chromatin 1 hour earlier than non-treated ones (data not shown; see Szöllösi et al., 1993).

Thus, an inhibition of MAP kinase activity following exit from metaphase II correlated with an advanced interphasic microtubule network formation. Furthermore, 6-DMAP was able to speed up moderately MAP kinase inactivation, probably by inhibiting upstream kinases.

DISCUSSION

The resumption of meiosis triggers a slow and stable activation of MAP kinase that differs from MPF activation

MAP kinase becomes active when phosphorylated on threonine and tyrosine residues (Anderson et al., 1990; Posada and Cooper, 1992). These phosphorylations can be assessed indirectly by MAP kinase mobility on SDS-PAGE. The phosphorylation of MAP kinase is associated with a slower mobility of the protein in all the systems so far examined. When MAP kinase is active it phosphorylates MBP with a high affinity. Our data show good correlations between MBP kinase activity in meiosis and MAP kinase mobility shifts. By an 'in gel' kinase assay, we were able to correlate this MBP kinase activity with two major proteins of 44 and 46×10^3 , the same M_r as the active forms of ERK 2 and ERK 1, respectively. All these criteria, combined with our previous results, suggest that the meiotic MBP kinase activity truly reflects MAP kinase activity during meiotic maturation.

Our results show striking differences in the kinetics of activation of H1 kinase and MAP kinase. First, H1 kinase is activated coincidentally with GVBD whereas MAP kinase activation is delayed until 2 hours later. Second, MAP kinase remains active whereas H1 kinase activity falls and rises again during the metaphase II to metaphase III transition, which is also the case during the natural MI/MII transition (Kubiak et al., 1992; Verlhac et al., 1993). Third, after egg activation, MAP kinase activity drops slowly for several hours after H1 kinase activity. In *Xenopus*, MAP kinase activity also persists during the MI to MII transition (Posada et al., 1991) and after activation of eggs by calcium. Furthermore, the inactivation of MAP kinase is also very slow and occurs after the drop in MPF activity (Nebreda and Hunt, 1993). However, in *Xenopus*, the rises in H1 kinase and MAP kinase activity occur at the same

time (Nebreda and Hunt, 1993). This suggests that the two enzymes may be activated by the same pathway in this species. In mouse oocyte, the two enzymes do not seem to be activated simultaneously. MAP kinase can be activated directly or indirectly by different kinases, such as the MAPKK or the product of the proto-oncogene *c-mos* (Nebreda and Hunt, 1993; Posada et al., 1993). However, we do not know when these kinases are activated during mouse meiotic maturation. We are studying presently the synthesis of the *c-mos* protein during maturation to determine if it is involved in the late activation of the MAP kinase. Since MAP kinase is one of the components of mouse meiotic spindle (Verlhac et al., 1993), this late kinase activation could be related to the slow meiotic spindle formation and the slow process of bivalent formation.

Does MAP kinase activity influence microtubule and chromatin organization?

We observed a close temporal correlation between the changes in microtubules and chromatin organization and MAP kinase activity during meiosis. First, MAP kinase becomes significantly active at around 3 hours after GVBD, coinciding with the formation of a bipolar spindle. Second, during the metaphase II to metaphase III transition, microtubules remain short and chromosomes stay condensed (Kubiak et al., 1992) and MAP kinase activity, but not MPF activity, stays high during this period. Third, after oocyte activation MAP kinase remains active until 2 hours after PBE. Concurrently, a genuine interphase microtubular array and decondensed chromosomes are not formed until 1.5 hours after PBE, despite a rapid drop in MPF activity occurring at polar body extrusion. Thus, kinases other than MPF are responsible for the maintenance of this metaphasic state. MAP kinase could be one of these kinases, since both MPF and MAP kinase can control microtubule dynamics in vitro (Verde et al., 1990; Gotoh et al., 1991b). Furthermore, a proportion of the MAP kinase is localized within the microtubule organizing centers (MTOCs) in mouse oocytes (Verlhac et al., 1993), suggesting that this enzyme could act in vivo on microtubule organization.

The treatment of activated mouse oocytes with 6-DMAP, a ser-thr kinase inhibitor, accelerates the transition to interphase (Szöllösi et al., 1993). We show here that 6-DMAP inhibits the MBP kinase activity when present in the in vitro assay (80%) and induces a small drop (around 25%) in MBP kinase activity associated with proteins of 44 and 46×10^3 after in vivo treatment. However, this inactivation is not associated with a shift in ERK 1 and ERK 2 mobilities. The shift in mobility becomes visible when eggs are incubated 1 hour longer in 6-DMAP. This could be explained in at least two ways. First, Posada and Cooper (1992) have shown that an inactive form of MAP kinase, only phosphorylated on threonine, has the same mobility as the active form when both threonine and tyrosine residues are phosphorylated. Thus, it is possible that a tyrosine dephosphorylation is induced by 6-DMAP, before the threonine dephosphorylation, with no visible shift in migration. Unfortunately, we cannot test this hypothesis because all the anti-phosphotyrosine antibodies tested so far were not sensitive enough. Second, the method used to detect the shift in mobility may possess a lower sensitivity compared to the measurement of MBP kinase activity. Nevertheless, the direct inhibition of MAP kinase by 6-DMAP coincides with an advanced interphasic microtubular network formation.

The formation of the interphasic microtubule network and the decondensation of chromatin take place before the complete inactivation of MAP kinase in both 6-DMAP-treated and non-treated eggs. This may suggest that even a moderate drop in MAP kinase activity is significant and induces the transition toward an interphasic morphological organization. Alternatively, MAP kinase may play a role only by maintaining a metaphasic organization whilst other enzyme(s), such as phosphatases, may favour the transition to interphase (Zernicka-Goetz et al., 1993).

MAP kinase is not active in mitosis

An MBP kinase activity is present during mitosis, although it is not observed in the in gel kinase assay and it is not associated with proteins of 44 and 46×10^3 . Similarly, we cannot detect any change in MAP kinase electrophoretic mobility during mitosis. These observations suggest that $p42^{\text{mapk}}$ and $p44^{\text{mapk}}$ are not responsible for the mitotic MBP kinase activity detected in vitro. The existence of an MBP kinase during mitosis in the absence of tyrosine phosphorylation of $p42^{\text{mapk}}$ has also been reported in *Xenopus* (Ferrel et al., 1991; Gotoh et al., 1991b) and in clam embryos (Shibuya et al., 1992). In clam embryos, a portion of the M-phase MBP kinase activity present during mitosis is related to *cdc2* (Shibuya et al., 1992). In our system, no drastic drop in MBP kinase activity is observed during the MII to MIII transition, when the histone H1 kinase activity drops down, suggesting that the MBP kinase present in mitosis in the mouse system is mostly not due to *cdc2*.

In different species, MAP kinase appears to be specifically activated during meiosis but not in mitosis (Ferrel et al., 1991; Gotoh et al., 1991b; Shibuya et al., 1992).

Role of MAP kinase activation during meiosis

MAP kinase is activated when quiescent somatic cells are stimulated by mitogenic factors and when prophase-arrested oocytes are stimulated to resume meiosis. This activation is only transient in the case of mitogen-stimulated cells and is also transient during clam oocyte maturation. After meiosis, clam eggs immediately enter the mitotic cell cycle without a meiotic arrest. By contrast, in *Xenopus* as well as in mouse oocytes, the metaphase II arrest occurs after the resumption of meiotic maturation. In both of these systems, MAP kinase remains active during the whole process of maturation and its inactivation follows the exit from this quiescent state (for review see Ruderman, 1993). It is possible that MAP kinase activation is triggered by exit from a quiescent state (in G_0 or in G_2) and its inactivation by an entry into a proliferative state (entry into the cell cycle or into a developmental program). Alternatively, but not exclusively, MAP kinase activity may be required for the transition between the two meiotic metaphases and for the remodelling of the sperm nucleus after fertilization. Thus, it would be of great interest to know the timing of MAP kinase inactivation in oocytes where fertilization occurs at metaphase I, such as certain molluscs.

Finally, MAP kinase may be involved in the maintenance of the second metaphase arrest occurring during meiotic maturation, and controlled by an activity called cytotostatic factor (CSF; Masui and Markert, 1971; Masui, 1991). The *c-mos* protein kinase, identified as an active part of CSF in amphibians (Sagata et al., 1989) is able to phosphorylate MAP kinase in

vitro, probably through MAPKK (Nebreda and Hunt, 1993; Posada et al., 1993). The potential role of MAP kinase in the maintenance of a metaphase-like state when MPF activity drops is consistent with MAP kinase being related to CSF.

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Note added in proof

The direct involvement of MAP kinase in the CSF activity was shown by Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E. and Maller, J. L. (1993) *Science* **262**, 1262-1265.