Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes

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In *Xenopus* oocytes, the *c-mos* proto-oncogene product has been proposed to act downstream of progesterone to control the entry into meiosis I, the transition from meiosis I to meiosis II, which is characterized by the absence of S phase, and the metaphase II arrest seen prior to fertilization. Here, we report that inhibition of Mos synthesis by morpholino antisense oligonucleotides does not prevent the progesterone-induced initiation of *Xenopus* oocyte meiotic maturation, as previously thought. Mos-depleted oocytes complete meiosis I but fail to arrest at metaphase II, entering a series of embryonic-like cell cycles accompanied by oscillations of Cdc2 activity and DNA replication. We propose that the unique and conserved role of Mos is to prevent mitotic cell cycles of the female gamete until the fertilization in *Xenopus*, starfish and mouse oocytes.

Keywords: *cdc2/mos/oocyte/proto-oncogene/Xenopus*

Introduction

In the ovaries of most animals, oocytes are arrested in prophase of the first meiotic division (prophase I) until a hormonal signal induces the transition from prophase I to an arrest point during the second meiotic division. This arrest is at metaphase II in *Xenopus*, mouse and most other vertebrates or at the pronucleus stage in starfish. The process that is initiated by hormonal stimulation, called meiotic maturation, is under the control of M-phase promoting factor (MPF), a ubiquitous complex between the Cyclin-dependent kinase Cdc2 and Cyclin B. In *Xenopus* prophase I arrested-oocytes, MPF is maintained in an inactive form by the phosphorylation of Cdc2 on Thr14 and Tyr15. In response to progesterone, Cdc2 is activated in a protein synthesis-dependent manner by the Cdc25 phosphatase, which removes the inhibitory phosphates of Cdc2. Active MPF induces germinal vesicle breakdown (GVBD), chromosome condensation and metaphase I spindle formation in oocytes. MPF activity falls after metaphase I, then rises again and remains high during metaphase II arrest until fertilization (Nebreda and Ferby, 2000). The second meiotic arrest is due to a cytostatic factor (CSF), which appears shortly after meiosis I and disappears after fertilization (Masui and Markert, 1971). Although a number of proteins have been

implicated as components of CSF activity, its biochemical composition is still unknown.

The *c-mos* gene was one of the first proto-oncogenes to be cloned (Oskarsson *et al.*, 1980). The synthesis of its product, the serine/threonine kinase Mos, is highly regulated and restricted in time and cell type. Mos is almost undetectable in somatic cells and is specifically expressed in germ cells, where it functions only during the short period of meiotic maturation before being proteolysed at fertilization. When ectopically expressed in somatic cells, Mos can induce either cell death or oncogenic transformation (Yew *et al.*, 1993). Mos is absent from prophase oocytes and is synthesized from maternal mRNA in vertebrates and starfish oocytes during meiotic maturation (Oskarsson *et al.*, 1980; Sagata *et al.*, 1988; Tachibana *et al.*, 2000). It activates a MAPK kinase (MEK), which in turn activates MAPK (Nebreda *et al.*, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). Ribosomal S6 kinase, Rsk, is then activated by MAPK (Palmer *et al.*, 1998). *Xenopus* Mos, as well as its downstream targets, are able to induce meiotic maturation in the absence of progesterone when microinjected into prophase oocytes (Yew *et al.*, 1992; Haccard *et al.*, 1995; Huang *et al.*, 1995; Gross *et al.*, 2001). Therefore, it has been proposed that Mos controls the entry into meiosis I. However, in mouse, starfish and goldfish, neither Mos synthesis nor MAPK activity are required for Cdc2 activation and progression through meiosis I (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1996; Sadler and Ruderman, 1998; Kajiura-Kobayashi *et al.*, 2000; Tachibana *et al.*, 2000). In contrast, in *Xenopus* oocytes, the destruction of Mos mRNA by antisense oligodeoxynucleotides was shown to prevent progesterone-induced GVBD (Sagata *et al.*, 1988). In addition, Mos is able to induce meiotic maturation when microinjected into *Xenopus* prophase oocytes, although it is not yet clear whether protein synthesis is needed for this to occur (Sagata *et al.*, 1989a; Yew *et al.*, 1992). Together, these results have led to the conclusion that Mos synthesis is both sufficient and required to initiate meiotic maturation. Consistent with this conclusion are observations that recombinant Mos was not able to activate Cdc2 in the presence of the MEK inhibitor, U0126, arguing that MAPK is the direct link between Mos and Cdc2 activation in *Xenopus* oocytes (Fisher *et al.*, 1999; Gross *et al.*, 2000). On the other hand, the prevention of MAPK activation by this inhibitor was recently shown to delay, but not to prevent, the Cdc2 activation induced by progesterone without affecting the synthesis of Mos. Therefore, progesterone appears to be able to activate Cdc2 by a mechanism that is independent of MAPK, a conclusion that is difficult to reconcile with a requirement for Mos downstream of progesterone in *Xenopus* oocyte. Two hypotheses could explain why Cdc2 activation is suppressed when Mos synthesis is prevented by antisense

oligonucleotides while it is not when MAPK activation is prevented by U0126 treatment. First, Mos could activate a MAPK-independent pathway. It has been recently proposed that Mos would downregulate Myt1, the inhibitory kinase of Cdc2, independently of MAPK (Peter *et al.*, 2002). However, this cannot explain why Mos is unable to induce Cdc2 activation in the absence of MAPK (Gross *et al.*, 2000). Secondly, the antisense oligonucleotides (Sagata *et al.*, 1988) could have effects other than just preventing Mos synthesis.

Besides the initial activation of Cdc2 prior to GVBD, Mos has been shown to be required during the metaphase I to metaphase II transition for the suppression of S phase. In *Xenopus* oocytes, when the synthesis or the activity of Mos is specifically inhibited at the time of GVBD, or when MAPK activation is prevented by U0126, the activity of Cdc2 remains low after completion of meiosis I, and oocytes fail to enter meiosis II (Daar *et al.*, 1991; Furuno *et al.*, 1994; Gross *et al.*, 2000). In these oocytes, Cyclin B does not re-accumulate, and both nuclear envelope reformation and DNA replication occur. However, in mouse oocytes, conflicting results have been obtained. Although the entry into meiosis II is not affected in oocytes from Mos^{-/-} mice (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1996), the injection of antisense oligonucleotides either arrests oocytes before the emission of the first polar body (Paules *et al.*, 1989) or induces nuclear reformation after meiosis I (O'Keefe *et al.*, 1989). Cyclin B does not re-accumulate and the activity of Cdc2 does not re-increase after GVBD (O'Keefe *et al.*, 1991), the same result seen following antisense oligonucleotide injections into *Xenopus* oocytes (Furuno *et al.*, 1994). Therefore, Mos ablation has different effects on meiotic maturation, depending on the experimental strategy. The most specific deletion method, gene knock-out, has been performed in mice and suggests that the essential role of Mos is to prevent parthenogenesis, a function that was not revealed by the antisense experiments performed in *Xenopus* and mouse oocytes.

At the end of meiotic maturation, Mos is thought to participate in the control of the second meiotic arrest of unfertilized eggs. Mos, as well as its downstream targets, exhibit CSF activity (Sagata *et al.*, 1989b; Haccard *et al.*, 1993; Huang *et al.*, 1995; Bhatt and Ferrell, 1999; Gross *et al.*, 1999), as defined by the original assay, which involves the microinjection into one blastomere of a two-cell embryo. When CSF is present, the injected blastomere arrests in metaphase, whereas the uninjected blastomere continues cell division (Masui and Markert, 1971). Nevertheless, the *in ovo* consequences of the ablation of Mos in unfertilized eggs has only been analyzed in mouse oocytes (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994).

In this study, we took advantage of a new experimental approach based on morpholino antisense oligonucleotides to gain new insights into the precise roles of Mos during *Xenopus* oocyte meiotic maturation. We were able to block Mos synthesis without inhibiting the entry into meiosis I and therefore were able to investigate the requirements for Mos during three key periods of meiotic maturation: the activation of Cdc2 before GVBD, the metaphase I to metaphase II transition characterized by the absence of DNA replication, and the metaphase II arrest. Here, we demonstrate that Mos synthesis is only required

after meiosis I, where it functions to prevent DNA synthesis and the spontaneous initiation of mitotic cell cycles, and to arrest oocyte meiosis in metaphase II, until fertilization.

Results

***Mos* ablation does not prevent GVBD and Cdc2 activation induced by progesterone**

To investigate the function of Mos synthesis in *Xenopus* oocytes, we have used a new approach based on morpholino antisense oligonucleotides. Morpholinos are oligonucleotides with a morpholine backbone. They have been shown to be very efficient gene-specific translational inhibitors in *Xenopus*, when designed as antisense oligonucleotides complementary to the sequence around the translation initiation codon (Heasman *et al.*, 2000). They prevent translation through steric blockade, rather than by targeting RNA for degradation, as DNA antisense oligonucleotides do (Summerton and Weller, 1997).

We compared the effects of conventional antisense DNA oligonucleotides (A⁻) with morpholino antisense oligonucleotides (M) on GVBD induced by progesterone. For both types of oligos, we had exactly the same sequence as the 25mer A⁻ oligonucleotide spanning the Mos mRNA start codon published in the pioneering paper by Sagata *et al.* (1988). For controls, we introduced six mispairs in the A⁻ sequence of the DNA and morpholino antisense oligonucleotides (Ac and Mc respectively), while preserving the same overall base composition.

As reported previously (Sagata *et al.*, 1988), the injection of antisense oligonucleotides completely inhibited progesterone-induced GVBD (Figure 1A). Surprisingly, the injection of morpholino antisense did not prevent GVBD in response to progesterone, although it was delayed by 2 h compared with control progesterone-treated oocytes. The morphology of the white spot at the animal pole of the cell was characteristic of oocytes that have undergone GVBD, and was identical in all maturing oocytes regardless of whether or not they had been injected with morpholino antisense (Figure 1A and see Figure 6). We directly ascertained that GVBD had really occurred in morpholino antisense-injected oocytes by oocyte dissection (Figure 1A). Control morpholinos or traditional oligos did not block maturation, although GVBD was delayed with control DNA oligonucleotides, suggesting that they have a non-specific effect. The amount of A⁻ oligonucleotide microinjected (130 ng) was chosen according to Sagata *et al.* (1988); a lower amount did not block GVBD in all oocytes, and higher amounts were known to be toxic. For morpholinos, we started from 130 ng per oocyte, and decreased the amount injected until we determined the lowest amount (84 ng per oocyte) that was able to completely block Mos accumulation in all experiments performed, using oocytes from 10 different frogs. The injections of morpholinos at amounts greater than 130 ng per oocyte were not toxic and did not inhibit GVBD in response to progesterone. The levels of Mos in injected oocytes were examined by western blotting, which confirmed that the protein was undetectable after injection with each type of antisense oligonucleotide (Figure 1B). The levels of Mos protein in both controls were always lower than in the uninjected

Fig. 1. Morpholino antisense inhibits Mos synthesis but does not prevent GVBD. (A) Prophase oocytes were injected or not (solid squares) with either 130 ng of antisense oligonucleotides A⁻ (open circles), 130 ng of control oligonucleotides Ac (solid circles), 84 ng of morpholino antisense M (open triangles) or 84 ng of control morpholino Mc (solid triangles). One hour after injection, oocytes were induced to mature by progesterone (Pg) and the percentage of GVBD was determined as a function of time by following the appearance of the typical white spot at the animal pole of the oocyte (left panel). Right panel: first row illustrates the external morphology of oocytes and second row illustrates sections of fixed oocytes (Pro, prophase oocyte; Pg, progesterone-matured oocyte at GVBD; Pg/M, morpholino antisense-injected oocyte treated by progesterone and fixed at GVBD). Arrowhead indicates the GV. (B) Western blots using *Xenopus* Mos antibody. One oocyte from the previous experiment was homogenized at GVBD and loaded in each lane. One prophase oocyte (Pro) was loaded on the first lane. The position of one molecular weight marker is indicated on the left. (C) Groups of 50 prophase oocytes were microinjected or not with morpholino antisense (M) and were metabolically labeled with [³⁵S]methionine at 200 μCi/ml. One hour later, progesterone (Pg) was added or not. Progesterone-treated oocytes were selected 4 h after GVBD. Mos immunoprecipitates were electrophoresed, transferred to nitrocellulose and first exposed to autoradiography (upper panel) and then probed with anti-Mos antibody (lower panel). The positions of molecular weight markers are indicated on the left.

progesterone-treated oocytes, perhaps indicating that mismatched pairs in a 25mer oligonucleotide might not be sufficient to completely abolish all of the antisense activity. The inhibition of Mos synthesis caused by morpholino antisense was further demonstrated by immunoprecipitation following [³⁵S]methionine labeling (Figure 1C). Taken together, our results demonstrate that GVBD is still induced by progesterone, despite the inhibition of Mos translation by morpholino antisense.

We next ascertained whether Cdc2 was normally activated by progesterone in the presence of morpholino

Fig. 2. Progesterone-induced Cdc2 activation in Mos-ablated oocytes. Oocytes were injected (C and D) or not (A and B) with morpholino antisense. One hour later (time 0), progesterone was added and oocytes were collected and homogenized at indicated times. eGVBD, first pigment rearrangement detected at the animal pole (15 min before GVBD); GVBD, well-defined white spot observed at 225 min in control oocytes and at 360 min in morpholino antisense-injected oocytes. The equivalent of three oocytes was assayed for H1 kinase activity (A and C). At indicated times, one oocyte was immunoblotted with antibodies against Tyr15-phosphorylated Cdc2 or against Cdc25 phosphatase (B and D). The positions of the molecular weight markers are indicated on the left.

antisense. The H1 kinase activity of Cdc2 in progesterone-treated oocytes peaked at the time of GVBD, regardless of whether or not oocytes had been injected with morpholinos (Figure 2A and C). In the absence of Mos, progesterone was therefore able to induce both Cdc2 activation and GVBD, although both events were delayed in comparison to control oocytes. Interestingly, this delay was never observed when maturation was induced by mRNA encoding the mouse Mos protein (data not shown). This suggests that although Mos is not required for maturation, it certainly contributes to the kinetics of GVBD and the rate of Cdc2 activation. As expected, the protein synthesis inhibitor, cycloheximide (CHX), was still able to prevent progesterone-induced maturation after morpholino injection (data not shown), indicating that the synthesis of proteins other than Mos is required to activate Cdc2. Since the initial activation of Cdc2 prior to GVBD depends on dephosphorylation of Cdc2 by the Cdc25 phosphatase, we monitored the phosphorylation state of

both proteins by western blotting (Figure 2B and D). In control and morpholino-injected oocytes, Cdc2 was fully dephosphorylated on Tyr15 in the absence of Mos, and Cdc25 was normally activated as shown by its electrophoretic shift at the time of GVBD (Figure 2B and D).

It has been reported previously that the absence of either Mos or MAPK activity prevents Cdc2 reactivation after metaphase I. In these conditions, Cyclins B1 and B2 fail to re-accumulate and the hyperphosphorylation of Cdc27, a component of the anaphase-promoting complex (APC) necessary for Cyclin B degradation, does not occur (Furuno *et al.*, 1994; Gross *et al.*, 2000). These results suggest that Mos and MAPK activity are required after GVBD for the accumulation of Cyclin B, perhaps through the regulation of the APC. H1 kinase activity was followed for 4 h after GVBD (Figure 2A), when normally maturing oocytes would progress through the meiosis I to meiosis II transition and arrest in metaphase II. Upon the exit from meiosis I, Cdc2 activity fell to a low level, due to Cyclin B degradation, and was then gradually reactivated during the 4 h period following GVBD in both the presence and absence of morpholino antisense (Figure 2A and C). Cdc25 underwent a partial and transient dephosphorylation in morpholino-injected oocytes, at the time of Cdc2 inactivation (Figure 2D). This observation suggests that, in the absence of Mos, a discrete Tyr phosphorylation of Cdc2 (see Figure 7A) might contribute to its inactivation after GVBD, a regulatory mechanism absent from control oocytes. We further analyzed the reactivation of Cdc2 after GVBD by monitoring the accumulation of Cyclins B1 and B2. In both control and morpholino antisense-injected oocytes, Cyclin B2 was shifted to a slower migrating form, reflecting Cdc2 activation by early GVBD (Figure 3A). The amounts of Cyclins B2 and B1 decreased after GVBD and subsequently increased again, although with a slower time course in morpholino-injected oocytes (Figure 3A and B). In addition, we analyzed Cdc27 phosphorylation level, as a representation of APC regulation (Gross *et al.*, 2000). At the time of GVBD, Cdc27 underwent a characteristic shift in its electrophoretic mobility (Figure 3C). This hyperphosphorylated form reappeared 2–3 h after GVBD, although with a delay in morpholino-injected oocytes (Figure 3C), in correlation with a slower Cyclin B accumulation (Figure 3A and B; Gross *et al.*, 2000). These results suggest that the absence of Mos influences the rate of Cyclin B degradation and re-accumulation.

Mos ablation prevents MAPK activation

Since even trace amounts of Mos are sufficient to activate MAPK, which could in turn activate Cdc2, it was important to ascertain that Mos did not re-accumulate during the 4 h period following GVBD in morpholino-injected oocytes. In progesterone-treated oocytes, Mos started to accumulate by early GVBD (eGVBD), in correlation with MAPK phosphorylation, visualized either by its electrophoretic retardation using an anti-MAPK antibody or by an anti-phospho-MAPK (Figure 4A and B). In contrast, in morpholino-injected oocytes, Mos remained undetectable and MAPK remained unphosphorylated (Figure 4A and B), even though MAPK was present at a constant level (Figure 4B). The absence of any MAPK activity was confirmed directly by an in-gel MBP kinase

Fig. 3. Cyclin B degradation is not affected in the absence of Mos. Oocytes were injected or not with morpholino antisense (M). One hour later (time 0), progesterone (Pg) was added and oocytes were homogenized at the indicated times. One oocyte equivalent was loaded in each lane and immunoblots were performed with antibodies against (A) *Xenopus* Cyclin B2, (B) *Xenopus* Cyclin B1 and (C) Cdc27. Oocyte lysates were originated from the experiment already described in Figure 2. The positions of the molecular weight markers are indicated on the left.

assay. As expected, a 42 kDa radioactive band appeared in uninjected control oocytes at GVBD and increased in intensity by 4 h after progesterone treatment (Figure 4C). Injection of morpholino antisense or addition of the MEK inhibitor, U0126, totally prevented the appearance of this radioactive band, even 4 h after GVBD (Figure 4C).

Rsk is partially activated in the absence of Mos and MAPK activity

In the absence of active MAPK, the injection of a constitutively active form of Rsk has been shown to restore Cyclin B accumulation after GVBD, leading to the reactivation of Cdc2 (Gross *et al.*, 2000). In morpholino-injected oocytes, Cdc2 reactivation occurred after GVBD, although MAPK was inactive. Since Rsk can be activated in the absence of any MAPK activity in other cell types (Kalab *et al.*, 1996; Jensen *et al.*, 1999), we evaluated the possibility that Rsk could still have been activated under our conditions. First, we looked at the electrophoretic mobility of Rsk, which is known to correlate with its phosphorylation and activation state (Figure 5A). In progesterone-treated oocytes, Rsk underwent an electrophoretic retardation starting at eGVBD (Figure 5A), in parallel with MAPK activation (Figure 4B). In morpholino-injected oocytes, Rsk exhibited only a partial shift of its electrophoretic mobility starting at eGVBD (Figure 5A).

Fig. 4. Mos ablation prevents MAPK activation induced by progesterone. Oocytes were injected or not with morpholino antisense (M) and incubated in the presence of progesterone (Pg) 1 h later (time 0). Oocytes were collected at indicated times and lysates originated from the experiment illustrated in Figure 2 were immunoblotted with the antibodies directed against (A) *Xenopus* Mos and (B) total MAPK or the active phosphorylated form of MAPK (P-MAPK). The positions of the molecular weight markers are indicated on the left. (C) In-gel assay of MBP kinase activities. Oocytes were injected or not with morpholino antisense (M) or incubated in the presence of 50 μ M U0126. One hour after injection or 20 min after incubation in U0126, progesterone was added (time 0) and oocytes were collected either at time 0 or at GVBD or 4 h after GVBD (240). MBP kinase activity was measured by an in-gel assay after adding (+MBP) or not (-MBP) myelin basic protein in the gel. The equivalent of one oocyte was loaded per lane. The positions of the molecular weight markers are indicated on the left.

We then directly assayed the activity of Rsk that had been immunoprecipitated 4 h after GVBD (Figure 5B). In morpholino-injected oocytes, Rsk activity was about half that of control oocytes, whereas it was completely inactive in oocytes injected with conventional A⁻ antisense oligonucleotides (Figure 5B). To ascertain whether or not the partial activation of Rsk was responsible for the initial activation of Cdc2 in response to progesterone, morpholino-injected oocytes were incubated in the presence of U0126, which is known to suppress Rsk activation in the oocyte (Gross *et al.*, 2000). Under these conditions, Cdc2 was still activated in response to progesterone, although Mos was not expressed and both MAPK and Rsk remained inactive (data not shown). Altogether, these results suggest that progesterone treatment leads to the activation of a U0126-sensitive pathway in *Xenopus* oocytes that is independent of Mos and active MAPK,

Fig. 5. Rsk is partially activated in the absence of Mos and MAPK activation. (A) Oocytes were injected or not with morpholino antisense (M). One hour later, progesterone (Pg) was added (time 0) and oocytes were homogenized at the indicated times. Oocyte extracts originated from the experiment illustrated in Figure 2 were immunoblotted with an antibody against Rsk2. (B) Oocytes were injected or not with either morpholino antisense (M) or traditional antisense (A⁻) oligonucleotides and were incubated in the presence of progesterone (Pg). Groups of 10 oocytes, either at prophase stage (Pro) or 4 h after GVBD (GVBD + 4 h) were homogenized and immunoprecipitated with the anti-Rsk2 antibody. Kinase activity was assayed using S6 peptide in immunoprecipitates.

but which can still lead to a partial activation of Rsk. It also indicates that the partially activated form of Rsk, if not necessary for the initial activation of Cdc2 induced by progesterone, could still be sufficient for Cdc2 re-activation after GVBD.

Mos is required to stabilize Cdc2 and to prevent DNA replication after meiosis I

Since *Xenopus* oocytes were able to mature in the absence of Mos when injected with morpholino antisense oligonucleotides, we next asked whether, in the absence of Mos, oocytes would arrest meiosis at metaphase II. We measured Cdc2 kinase activity after meiosis I, at a time when control uninjected oocytes had already arrested at metaphase II, as a result of CSF activity. In such uninjected progesterone-treated oocytes, Cdc2 activity reappeared after GVBD and stabilized at a high level for at least 5 h (Figure 6A). In contrast, in Mos-ablated oocytes, Cdc2 activity started to fluctuate after its initial re-appearance following GVBD, alternating between high and low activity during the following 5 h (Figure 6A). As described above, in these Mos-ablated oocytes, the activity of Rsk was half that of the control progesterone-treated oocytes (Figure 6A). The external morphology of Mos-depleted oocytes also started to change 3 h after GVBD by exhibiting a pigment rearrangement similar to those seen after the parthenogenetic activation of eggs (Figure 6A,

Fig. 6. Mos is required to stabilize Cdc2 activity after GVBD. (A) Oocytes were injected (solid squares, solid bars) or not (open squares, open bars) with morpholino antisense (M). One hour later (time 0), progesterone was added. GVBD started 4 h after progesterone addition in control oocytes, and with a 4 h delay in morpholino antisense-injected oocytes. At GVBD, some morpholino antisense-injected oocytes were injected with 50 ng of recombinant MBP-Mos protein (hatched bar). At indicated times, oocytes were homogenized and kinase activities of Cdc2 (H1 phosphorylation: lines) and Rsk (S6 peptide phosphorylation: bars) were assayed. Each point of Cdc2 and Rsk activities corresponds to four oocytes. The external morphology of injected oocytes is illustrated at the time of GVBD (a) and 5.5 h after GVBD with (b) or without (c) MBP-Mos injection at GVBD. (B) Oocytes were injected (solid squares) or not (open squares) with morpholino antisense (M). One hour later (time 0), progesterone was added. GVBD started 4 h after progesterone addition in control oocytes, and with 3 h delay in morpholino antisense-injected oocytes. At GVBD, some Mos-ablated oocytes were injected with 50 ng of recombinant MBP-Mos protein (triangles). At indicated times, oocytes were homogenized and Cdc2 kinase activity was assayed (three oocytes per point).

panel c). During this post-GVBD period, Mos was absent and MAPK activity was undetectable (data not shown). In Mos-ablated oocytes, the injection of *Xenopus* MBP-Mos protein at GVBD was sufficient to restore a full level of Rsk activity (Figure 6A) and a full-sustained level of Cdc2 activity (Figure 6B). Moreover, it prevented the abnormalities of the oocyte external morphology (Figure 6A, panel b), demonstrating that our observations were a consequence of Mos ablation. An identical rescue of Mos-ablation defects was obtained by microinjection of mouse Mos mRNA, which lacks the 5' UTR-specific target sequence for morpholino antisense (data not shown).

After meiosis I, Mos-ablated oocytes could either progress through meiotic-like cell cycles, consisting of consecutive M phases without intervening S phase, or start mitotic-like cell cycles characterized by an alternation of S

Fig. 7. Analysis of Cyclin B2 levels, Cdc2 phosphorylation and DNA replication in Mos-ablated oocytes after GVBD. (A) Oocytes were injected or not with morpholino antisense (M). Six hours later, progesterone (Pg) was added (time 0). Some of the progesterone-treated oocytes were incubated 45 min after GVBD in CHX. Oocytes were collected at indicated times and immunoblotted with antibodies against *Xenopus* Cyclin B2 and Tyr15-phosphorylated form of Cdc2. GVBD occurred 5 h after progesterone addition in control oocytes and 3 h later in morpholino antisense-injected oocytes. (B) In parallel, 1 h after progesterone addition, oocytes were injected with [α -³²P]dCTP (50 nM, 3000 Ci/mmol). Aphidicolin (APD) was added at the time of GVBD and CHX 45 min after GVBD. Five hours after GVBD, DNA was extracted and incorporation of radioactive dCTP was visualized by autoradiography. Each lane corresponds to the equivalent of one oocyte. Prophase oocytes (Pro) were also injected with [α -³²P]dCTP and collected 10 h after GVBD occurring in the control oocytes.

and M phases. We therefore analyzed Cyclin B2 levels (Figure 7A) and DNA synthesis (Figure 7B) in control and Mos-ablated oocytes. In progesterone-treated control oocytes, Cyclin B2 re-accumulated rapidly after meiosis I and remained stable throughout the metaphase II arrest (Figure 7A). In the absence of Mos, levels of Cyclin B2 strongly decreased after GVBD, re-accumulated after a delay and to lower levels than in control oocytes, peaked at 300 min after GVBD, and then decreased again. A faster migrating form of Cyclin B2 was detected during the period between 180 and 240 min (in the experiment illustrated in Figure 7A), due to its association with a form of Cdc2 that was phosphorylated on Tyr15 (Figure 7A and see also the Cdc25 phosphorylation level in Figure 2D). We then investigated DNA replication by measuring the incorporation of radioactive deoxycytidine triphosphate (dCTP) into genomic DNA, 300 min after GVBD. As shown in Figure 7B, prophase oocytes and control metaphase II-arrested oocytes did not incorporate radioactive dCTP. Inhibition of protein synthesis shortly after GVBD induced Cyclin B2 degradation (Figure 7A), the entry into interphase and the formation of replicative nuclei in the oocytes (Huchon *et al.*, 1993; Furuno *et al.*,

1994). As expected, progesterone-treated oocytes underwent DNA replication when CHX was added shortly after GVBD, providing a positive control (Figure 7B). Morpholino-injected oocytes incorporated radioactive dCTP to the same extent as CHX-treated oocytes (Figure 7B). In both experiments, aphidicolin, a specific inhibitor of DNA polymerase α (Furuno *et al.*, 1994), abolished dCTP incorporation (Figure 7B). Therefore, in the absence of Mos, oocytes do not complete meiosis and instead directly enter in mitotic cell cycles.

Discussion

One of the key regulators of meiosis is the product of the proto-oncogene *c-mos*, which is specifically expressed in germ cells and functions only during meiotic maturation. In this study, we used morpholino antisense oligonucleotides to inhibit Mos translation induced by progesterone without preventing the initiation of meiotic maturation. Because this new experimental approach does not block the initial activation of Cdc2 induced by progesterone, we were able to investigate the exact requirements for Mos during the three periods of *Xenopus* oocyte meiotic maturation: the entry into meiosis I, the metaphase I to metaphase II transition characterized by the absence of S phase, and the metaphase II arrest. Here, we provide evidence that progesterone is able to activate a pathway independent of Mos synthesis leading to Cdc2 activation and GVBD. After GVBD, Mos is also dispensable for the activation of Cdc2 and the accumulation of Cyclin B, providing that Rsk is active. However, in the absence of Mos, the activity of Cdc2 is not stabilized. Cyclin B levels oscillate, Cdc2 is partially rephosphorylated on tyrosine, and genomic DNA undergoes replication. This series of events mimic the mitotic cell cycles of the early embryos.

It has been shown, 14 years ago by microinjection of conventional antisense oligonucleotides, that Mos synthesis is required for Cdc2 activation in response to progesterone. On the other hand, two reports recently showed that MAPK, the target of Mos, is not necessary for Cdc2 activation after progesterone stimulation (Fisher *et al.*, 1999; Gross *et al.*, 2000). In the present work, we used morpholino antisense oligonucleotides to clarify these contradictory results by inhibiting both Mos synthesis and the downstream activation of MAPK, as well as other unknown targets of Mos. Our results show that Cdc2 can be activated in *Xenopus* oocytes by a pathway that does not require Mos synthesis or elevated MAPK activity. These results are consistent with results in starfish and mammals (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Tachibana *et al.*, 2000). Therefore, although exogenous Mos is able to induce GVBD, it is not necessary and the synthesis of new unidentified protein(s) is still required to control Cdc2 activation after progesterone treatment. An attractive candidate as a protein neosynthesized in response to progesterone and necessary for Cdc2 activation is the RINGO/Speedy protein, a non-conventional activatory partner of Cdc2 (Ferby *et al.*, 1999; Lenormand *et al.*, 1999; Karaïskou *et al.*, 2001). The reproducible delay observed in Cdc2 activation was rescued in morpholino-injected oocytes by the injection of Mos mouse mRNA (data not shown), indicating that the Mos/MEK/MAPK/Rsk pathway contributes to the kinetics of MPF activation,

as described previously (Fisher *et al.*, 1999; Gross *et al.*, 2000, 2001). This contribution is probably achieved through the inhibition of a negative regulator of Cdc2, the Myt1 kinase. Myt1 could be downregulated directly by Rsk (Palmer *et al.*, 1998), by Akt as reported for starfish oocytes (Okumura *et al.*, 2002) or by Mos as suggested by Peter *et al.* (2002). According to Peter *et al.* (2002), the absence of Mos leads to a delayed Myt1 inactivation, explaining why Cdc2 activation, hence GVBD, are delayed in morpholino-injected oocytes. Nevertheless, the Mos protein and the MAPK/Rsk activities remain dispensable since their suppression by morpholino antisense or by U0126, respectively, prevents neither Cdc2 activation nor GVBD induction in response to progesterone (Gross *et al.*, 2000; our data).

Some hypotheses could be postulated to explain why the response is different when morpholino oligonucleotides are used for Mos ablation instead of conventional oligonucleotides. Morpholinos block translation by an RNase-H-independent mechanism leaving the target mRNA intact (Summerton and Weller, 1997), while conventional oligonucleotides induce RNase-H cleavage of the mRNA, generating 3' and 5' fragments (Ballantyne *et al.*, 1997). The possibility that these fragments prevent the synthesis of protein(s) required for meiotic maturation non-specifically, through a general inhibition of translation, cannot be excluded. Another interesting possibility is that the mRNA encoding Mos could itself play a role in the meiotic maturation. The degradation of the mRNA induced by the injection of conventional oligonucleotides could, for example, lead to the release of some regulatory RNA-binding proteins such as OMA1 and OMA2, which have been reported to function upstream of Myt1 in *Caenorhabditis elegans* (Detwiler *et al.*, 2001). Furthermore, the RNase-H action could generate new stable mRNA fragments encoding for N-terminal-truncated proteins, as has been recently described (Thoma *et al.*, 2001). These proteins could interfere with meiotic maturation. Therefore, there are a variety of potential mechanisms by which conventional DNA antisense oligonucleotides targeted to different regions of the Mos mRNA could block progesterone-induced meiotic maturation in *Xenopus*. However, our results clearly show that Cdc2 activation and GVBD can take place in the absence of Mos synthesis. Hence, our results resolve the long-standing inconsistencies concerning the roles of Mos during oocyte maturation that were deduced from studies in different species. Moreover, our findings completely revise the view that Mos is an essential component of the progesterone signaling pathway leading to the initial activation of Cdc2 in *Xenopus* oocytes. The crucial function of the Mos/MAPK cascade now appears much more similar in animal species than thought previously, and seems to be a consequence of the Cdc2 activation rather than a Mos-dependent trigger of Cdc2 activation (Nebreda *et al.*, 1995; Frank-Vaillant *et al.*, 1999). Nevertheless, this pathway must play a role during metaphase I, most probably at the level of spindle assembly or dynamics (Zhou *et al.*, 1991; Verlhac *et al.*, 1994, 1996; Choi *et al.*, 1996).

When MAPK and Rsk activation is prevented by U0126, the initial activation of Cdc2 occurs, and Mos accumulates in response to progesterone (Gross *et al.*,

2000). However, after meiosis I, Cyclin B fails to re-accumulate, and both Cdc2 and Rsk remain inactive despite the presence of Mos. Oocytes then fail to reach meiosis II and instead enter an interphase-like stage. The injection of a constitutively active Rsk restores Cyclin B protein levels, the post-GVBD reactivation of Cdc2 and the metaphase II arrest (Gross *et al.*, 2000). In contrast, in morpholino-injected oocytes, the accumulation of Cyclin B after GVBD is not impaired and Cdc2 reappears. This difference is probably due to the fact that Rsk is partially activated after the Mos ablation by morpholinos, while it is never activated after U0126 treatment. The high concentrations of U0126 (50 μ M) required to inhibit MAPK activation in the amphibian oocyte (Gross *et al.*, 2000) could explain the differences in Rsk activity seen under these experimental conditions. The U0126 concentrations used in these experiments would be expected to inhibit the desired targets, MEK1/2, but also other protein kinases such as p70^{S6K}, PRAK, PKB or p38 (Davies *et al.*, 2000). Consequently, other pathways, including a Mos-independent pathway that activates Rsk, would be blocked in addition to the MEK/MAPK pathway. This interpretation is supported by our observations that the addition of U0126 to morpholino-injected oocytes totally abolishes Rsk activity. Therefore, an alternative pathway, independent of the Mos/MAPK pathway but sensitive to 50 μ M U0126, may be involved in the partial activation of Rsk. In mouse meiotic maturation, a partial phosphorylation and activation of Rsk has been reported in the absence of Mos and MAPK activity (Kalab *et al.*, 1996). In this species, two sequentially activated pathways contribute to Rsk activation. The first level of phosphorylation and activation of Rsk is reached 1 h after GVBD, before any MAPK activation. The full activation of Rsk is then achieved 1 h later, at the time of MAPK activation (Kalab *et al.*, 1996). In mammalian cells, it has been demonstrated that the level of Rsk activity depends on a balanced input from two kinases: PDK1 (3-phosphoinositide-dependent kinase-1) and MAPK (Jensen *et al.*, 1999). Rsk is composed of two kinase domains called N-terminal kinase (NTK) and C-terminal kinase (CTK), separated by a linker region. PDK1 phosphorylates and activates the NTK domain (Jensen *et al.*, 1999; Richards *et al.*, 1999), while MAPK phosphorylates the linker and the CTK domain, leading to its activation (Fisher and Blenis, 1996; Dalby *et al.*, 1998). Although MAPK is required for the full activation of Rsk, the phosphorylation of full-length Rsk2 by ectopic PDK1 induces its partial activation, without the involvement of MAPK (Jensen *et al.*, 1999). In addition, Rsk cannot be activated by MAPK in the absence of PDK1 activity, suggesting that PDK1 and MAPK should act hierarchically (Jensen *et al.*, 1999). A PDK1-like activity in *Xenopus* oocytes is likely to be responsible for the partial activation of Rsk observed in Mos-depleted oocytes. *In vitro*, it has been demonstrated that a CTK-deleted Rsk, whose activity depends only on its phosphorylation by PDK1, was partially activated by human PDK1 (Gross *et al.*, 1999). This construct was activated during incubation into *Xenopus* oocyte M phase extracts, but poorly in prophase extracts (Gross *et al.*, 2001), suggesting that this PDK1-like activity is cell cycle regulated.

In *Xenopus*, the CSF activity of Mos was demonstrated by the injection of either mRNA or recombinant protein

into a two-cell stage embryo resulting in a cell cycle arrest at metaphase in the injected blastomere (Sagata *et al.*, 1989b). Here, for the first time, we were able to follow the whole maturation process in the absence of Mos and, thus, to study the behavior of a Mos-ablated *Xenopus* oocyte during the transition from meiosis I to meiosis II. Our results show that Cdc2 kinase activity cycles and cannot be stabilized after GVBD in the absence of Mos. Cdc2 is transiently rephosphorylated on tyrosine, Cyclin B levels oscillate and oocytes replicate DNA after meiosis I. We were unable to detect any meiotic spindle or polar body extrusion in Mos-ablated oocytes (data not shown). Therefore, in the absence of Mos and despite the presence of partially active Rsk, oocytes fail to undergo a CSF arrest at metaphase II, and instead enter a series of mitotic-like cell cycles. This behavior is similar to the one described in Mos-ablated starfish oocytes, which undergo 3–4 Cdc2 cycles after meiosis I (Tachibana *et al.*, 2000). The primary effect of the Mos/MAPK pathway might be to regulate Cdc2 activity during the interkinesis between metaphase I and metaphase II. When present, this pathway could prevent the inactivation of Cdc2 by tyrosine phosphorylation or could decrease the efficiency of APC towards Cyclin B. It is highly possible that this link between the Mos/MAPK/Rsk pathway and the Cyclin B degradation machinery (APC) could involve the new Cdc20 binding protein, Emi1 (Reimann *et al.*, 2001; Reimann and Jackson, 2002). It could also stimulate Cyclin B translation, leading to a precocious reactivation of Cdc2 required for the entry into metaphase II without interphase. How Mos and MAPK are molecularly coupled either to the control of the Cyclin mRNA translation, or to the regulators of Cdc2 phosphorylation, Myt1/Wee1 and Cdc25, then represents an important aim. Moreover, it has been clearly described that the Mos/MAPK pathway regulates the meiotic spindle organization in mouse oocytes (Choi *et al.*, 1996; Verlhac *et al.*, 1996). This pathway is also certainly implicated in *Xenopus* oocytes, since no spindle and no polar body extrusion were detected in the absence of Mos or active MAPK (Gross *et al.*, 2000; our data). Because in Mos-ablated oocytes a normal phenotype was totally restored by the reinstatement of recombinant Mos or by the *in vivo* translation of morpholino-resistant Mos mRNA, this protein could be considered as necessary and sufficient to prevent premature mitotic cell cycles until fertilization. These observations are different from what has been described previously in mouse oocytes. Although Mos^{-/-} mice oocytes do not arrest in metaphase II (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1996), Cdc2 activity does not oscillate, and 76% of oocytes arrest at a 'metaphase III' stage (Verlhac *et al.*, 1996). In addition, Mos^{-/-} oocytes do not replicate DNA between metaphase I and metaphase II, despite going through an interphase-like stage (Verlhac *et al.*, 1996). This is probably because mouse oocytes do not acquire functional replicative machinery before metaphase II, in contrast to starfish and *Xenopus* oocytes that develop the ability to replicate DNA early after GVBD (Furuno *et al.*, 1994; Tachibana *et al.*, 1997). The spindle checkpoint, defective in starfish and *Xenopus* embryos (Hara *et al.*, 1980; Gerhart *et al.*, 1984; Vee *et al.*, 2001), but active in both mouse oocytes and embryos (Kubiak *et al.*, 1993; Winston *et al.*, 1995),

probably ensures the ‘metaphase III’ arrest in mouse oocytes and could also account for these inter-species differences.

In conclusion, the specific role that Mos plays in meiosis is the prevention of mitotic cell cycles after meiosis I and the generation of a cell cycle-arrested gamete awaiting fertilization. This role now appears to be universal, since it is found in oocytes from organisms as diverse as *Xenopus*, starfish, goldfish and mice.

Materials and methods

Materials

Xenopus laevis adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions. [γ - 32 P]ATP, [α - 32 P]dCTP and [35 S]methionine were purchased from Dupont NEN (Boston, MA). Reagents, unless otherwise specified, were from Sigma (Saint Quentin Fallavier, France). Morpholinos and conventional DNA oligonucleotides were purchased from Gene Tools LLC and Eurogentec, respectively. The sequence of both antisense morpholinos and conventional DNA oligonucleotides used was AAGGCATTGCTGTGTGACTCGCTGA, as described previously (Sagata *et al.*, 1988), and the sequence of both control morpholinos and ‘traditional’ DNA oligos used was TAGACA-ATGCTCTGTGGCTCGGTGA. Recombinant MBP-Mos protein was bacterially expressed and prepared as described previously (Roy *et al.*, 1996).

Preparation and handling of oocytes

Fully grown *Xenopus* prophase oocytes were obtained as described by Jessus *et al.* (1987). The usual microinjected volume was 50 nl per oocyte. Progesterone and U0126 (Promega) were used in the external medium at final concentrations of 2 and 50 μ M, respectively. Oocytes were referred as to eGVBD when the first pigment rearrangement was detected at the animal pole, and as GVBD when a well-defined white spot was formed. In all experiments, GVBD was ascertained by dissection of TCA-fixed oocytes. For extract preparations, oocytes were lysed in 10 vols of EB [80 mM β -glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 1 mM dithiothreitol (DTT) pH 7.3] supplemented with protease inhibitor cocktail (P8340, Sigma) and then centrifuged at 15 000 g for 10 min at 4°C.

Antibodies, western blotting and immunoprecipitations

Western blots were performed as described (Frank-Vaillant *et al.*, 1999). The antibodies used were: rabbit polyclonal antibody raised against the *Xenopus* Mos protein (Santa Cruz Biotechnologies) for western blotting, monoclonal mouse anti-Mos antibodies (R38 and R97, a kind gift from Dr J.Gannon, ICRF, UK) for immunoprecipitation, sheep polyclonal antibodies raised against *Xenopus* Cyclin B2 or B1 and rabbit polyclonal antibody against Cdc25 (kind gifts from Dr J.Maller, HHMI, CO), rabbit polyclonal antibodies against Tyr15-phosphorylated Cdc2 (Cell Signaling Technology, Inc.), mouse monoclonal antibody against Cdc27 (Santa Cruz Biotechnologies), rabbit polyclonal antibodies against total ERK (Santa Cruz Biotechnologies), mouse monoclonal antibodies against the active phosphorylated form of MAPK, P-MAPK (New England Biolabs) and rabbit polyclonal antibodies against Rsk2 (Santa Cruz Biotechnologies). The appropriate horseradish peroxidase-labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA) were revealed by chemiluminescence (NEN). Immunoprecipitations of Mos and Rsk were performed according to Nebreda *et al.* (1995) and Gross *et al.* (1999), respectively.

Kinase assays

Cdc2 activity (equivalent to three or four oocytes) was assayed using histone H1 as a substrate after affinity purification on p13^{suc1} beads as described (Jessus *et al.*, 1991). MBP kinase activity was measured by an in-gel assay after adding or not myelin basic protein (MBP from bovine brain, Sigma) in the electrophoresis gels, as described (Durocher and Chevalier, 1994). For the Rsk activity assay, lysates from 4–10 oocytes were immunoprecipitated with the anti-Rsk2 antibody, and the kinase assay was performed in immunoprecipitates in the presence of 250 μ M S6 peptide (kind gift from Dr J.Goris, Belgium), as described previously (Gross *et al.*, 1999).

Analysis of DNA synthesis

[α - 32 P]dCTP (3000 Ci/mmol, 10 mCi/ml) was injected 1 h after progesterone addition. For analysis, 10 oocytes were lysed in homogenization buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS) and treated with proteinase K (100 μ g/ml) at 55°C overnight. After successive extractions by an equal volume of phenol, phenol/chloroform and chloroform, DNA was precipitated by 0.6 vols of isopropanol at –20°C and re-suspended in 100 μ l of TE (10 mM Tris pH 7.5, 1 mM EDTA). After treatment by RNase T1 (10 μ g/ml) for 1 h at room temperature, samples were submitted to 0.9% agarose gel electrophoresis (one oocyte equivalent per lane). Incorporation of radioactive dCTP was revealed by autoradiography after migration. CHX (10 μ g/ml) was added to the medium within 45 min after GVBD, and aphidicolin (20 μ g/ml) at the time of GVBD, before detectable DNA synthesis occurred in CHX-treated controls.

Acknowledgements

We thank Dr T.Hunt for anti-Mos antibodies and valuable advice, Dr J.Goris for S6 peptide, Dr E.Houliston, Dr A.Karaïskou, Dr M.-H.Verlhac and Dr M.Lohka for critical reading of the manuscript, Dr J.Maller for anti-Cdc25, Cyclins B1 and B2 antibodies, Dr M.-H.Verlhac for mouse RNA and all the members of the laboratory for their support. This work was supported by grants from INRA, CNRS, University Pierre and Marie Curie, ARC (No. 5899 to C.J.) and IFR-BI (to O.H.).

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*Received March 1, 2002; revised June 6, 2002;
accepted June 7, 2002*