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MAPK inactivation is required for the G_2 to M-phase transition of the first mitotic cell cycle

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Down-regulation of MAP kinase (MAPK) is a universal consequence of fertilization in the animal kingdom, although its role is not known. Here we show that MAPK inactivation is essential for embryos, both vertebrate and invertebrate, to enter first mitosis. Suppressing down-regulation of MAPK at fertilization, for example by constitutively activating the upstream MAPK cascade, specifically suppresses cyclin B–cdc2 kinase activation and its consequence, entry into first mitosis. It thus appears that MAPK functions in meiotic maturation by preventing unfertilized eggs from proceeding into parthenogenetic development. The most general effect of artificially maintaining MAPK activity after fertilization is prevention of the G_2 to M-phase transition in the first mitotic cell cycle, even though inappropriate reactivation of MAPK after fertilization may lead to metaphase arrest in vertebrates. Advancing the time of MAPK inactivation in fertilized eggs does not, however, speed up their entry into first mitosis. Thus, sustained activity of MAPK during part of the first mitotic cell cycle is not responsible for late entry of fertilized eggs into first mitosis.

Keywords: cyclin B–cdc2 kinase/fertilization/G_2 to M-phase transition/MAP kinase inactivation/mitosis

Introduction

In the animal kingdom, the cell cycle proceeds very rapidly during early embryogenesis, and consists only of alternating S- and M-phases, without intervening gap phases. In many species, both vertebrate and invertebrate, the first mitotic cell cycle after fertilization is exceptionally long compared with the following ones. The increased length of the first mitotic cell cycle is only partly accounted for by a reduced rate of DNA replication. In the fertilized *Xenopus* egg at 20°C, for example, DNA replication starts 29 min after fertilization, before pronuclei fusion, and it is completed within the next 23 min (Gerhart, 1980). Nonetheless, the zygote nucleus envelope does not break down earlier than 69 min after fertilization. The interval of time from completion of the first round of DNA replication to initiation of the second one is about 34 min, whilst the total length of the 11 forthcoming cell cycles is as short as 20 min. This is due in part to the presence of a 17 min G_2 phase in the first mitotic cell cycle only. As observed in oocytes and somatic cells, G_2 phase is associated with tyrosine phosphorylation of cdc2 and inactivation of cyclin B–cdc2 kinase. However, the mechanism responsible for tyrosine phosphorylation of cdc2 in the first mitotic cell cycle only remains elusive.

Mitogen activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) are activated through a cascade of conserved kinases in response to a variety of extracellular signals including growth factors and hormones, and couple extracellular stimuli to transcriptional activation in somatic cells (reviewed by Karin and Hunter, 1995; Waskiewiez and Cooper, 1995). MAPKs are also activated during meiotic maturation and have been shown to play a role in mechanisms controlling MPF activity (reviewed by Kosako *et al.*, 1994; Sagata, 1997). At variance with the first mitotic cell cycle that follows fertilization, the 11 forthcoming ones proceed in the absence of MAPK activity in *Xenopus* embryos, and MAPK is reactivated only when intervening gap phases reappear in the cell cycle (Ferrell *et al.*, 1991). MAPK activity also drops during the first mitotic cell cycle in mammals, molluscs and echinoderms, and does not reappear during the forthcoming early ones which proceed more rapidly than the first one (Shibuya *et al.*, 1992; Verlhac *et al.*, 1994; Picard *et al.*, 1996).

We recently reported that fertilized eggs of the starfish *Marthasterias glacialis* microinjected with a constitutively active Ste 11–ΔN mutant, a MAPK kinase kinase that clamps MAPK at a high level of activity, replicate DNA, but fail to enter mitosis and thus arrest at G_2 (Picard *et al.*, 1996). Mature oocytes of the same starfish species replicate DNA after second polar body emission, then arrest with high MAP kinase activity if not fertilized. Taken together, these observations suggested that MAPK, which is active during part of the first, slow mitotic cell cycle, inactive during the forthcoming rapid ones and again reactivated later on when intervening gap phases reappear, may act as a brake on cell cycle progression.

The aim of the present study was to examine this possibility. We find that MAPK inactivation is essential for embryos to enter, then to complete first mitosis. However, advancing the time of MAPK inactivation after fertilization does not speed up the schedule for entry into mitosis, thus MAPK is not responsible for the increased length of the first cell cycle as compared with the following ones in the early embryo. The possibility that unrestrained activity of MAPK might inhibit progression of somatic cells into mitosis, as it does in embryonic cells, is discussed.

Results

MAPK inactivation is required for Xenopus eggs to enter first mitosis

MAPK has been shown to be inactivated before entry into first mitosis in fertilized or parthenogenetically-activated

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eggs, but whether this inactivation is actually required for progression into mitosis has not been investigated. We have previously reported that activation of MAPK through addition of recombinant c-mos in interphase egg extracts suppresses activation of the cyclin degradation pathway, but not the ability of recombinant cyclin B to form active complexes with endogenous cdc2 (Abrieu *et al.*, 1996). However, in these experiments the use of recombinant cyclin B in excess could have displaced an equilibrium (Solomon *et al.*, 1990) and prevented detection of a MAPK-dependent block to cyclin B–cdc2 kinase activation. Indeed we have found that microinjection of the same recombinant c-mos protein in progesterone-matured *Xenopus* oocytes suppressed the reappearance of cdc2 kinase activity after its drop following parthenogenetic activation (data not shown; Karsenti *et al.*, 1987).

In contrast, in another *in vitro* experiment no exogenous cyclin was added, and translation from mRNA of endogenous cyclins was used to drive the cell cycle from interphase into mitosis. Part of the cycling extract was clamped with high MAPK activity by adding to the extract, soon after its preparation, a non-degradable c-mos or Ste 11–ΔN fusion protein, both acting as potent MAPKKK (Figure 1A). Under these conditions, pronuclei assembled and replicated DNA, as observed in control cycling extracts devoid of MAPK activity (Figure 1B and C).

However, pronuclei neither condensed chromosomes nor underwent nuclear envelope breakdown (NEBD). Instead, the G_2-arrested pronuclei continued to enlarge after completion of DNA replication, to reach, after 2 h, a diameter at least 2-fold that of control pronuclei at the time of NEBD, i.e. ~15 min after completion of DNA replication (Figure 1B). Similar results were obtained by others, using a Ras-leu 61 mutant to arrest *Xenopus* egg extracts at interphase (Chen and Pan, 1994 ; Pan *et al.*, 1994).

We also investigated the time course of H1 histone kinase activity in anti-cyclin A and anti-cyclin B immunoprecipitates prepared from the same extracts. Cyclin A is exclusively associated with cdc2 in the early *Xenopus* embryo (Roy *et al.*, 1991; Devault *et al.*, 1992; Rempel *et al.*, 1995), thus only cdc2 kinases activities were monitored in these experiments. As shown in Figure 2A, cyclin A–cdc2 kinase was activated with roughly the same kinetics in extracts containing *c-mos* and in control extracts. However, cyclin A–cdc2 kinase did not undergo degradation in extracts clamped with high MAPK activity, whilst it dropped abruptly after 50 min in control cycling extracts, due to cyclin A degradation at prometaphase. In contrast to cyclin A–cdc2 kinase, cyclin B1–cdc2 kinase was not normally activated in extracts containing high MAPK activity, although it was activated very late and to a limited extent in some extracts as compared with control cycling extracts (Figure 2B). Similar results were obtained when H1 kinase was measured in anti-cyclin B2–cdc2 kinase (Figure 2C), which activates later on and more abruptly in cycling extracts, as it does in the fertilized egg (Minshull *et al.*, 1990).

To establish that suppression of cyclin B–cdc2 kinase was actually due to MAPK activity in the above experiments, not merely to the toxic effect of adding a protein of bacterial origin or to some unexpected property of c-mos, the same experiments were repeated, but the CL100 protein phosphatase (Alessi *et al.*, 1993) was added simultaneously with c-mos to suppress MAPK activity. As shown in Figure 2D, CL100 completely suppressed the inhibitory effect of c-mos and restored cyclin B–cdc2 kinase activation.

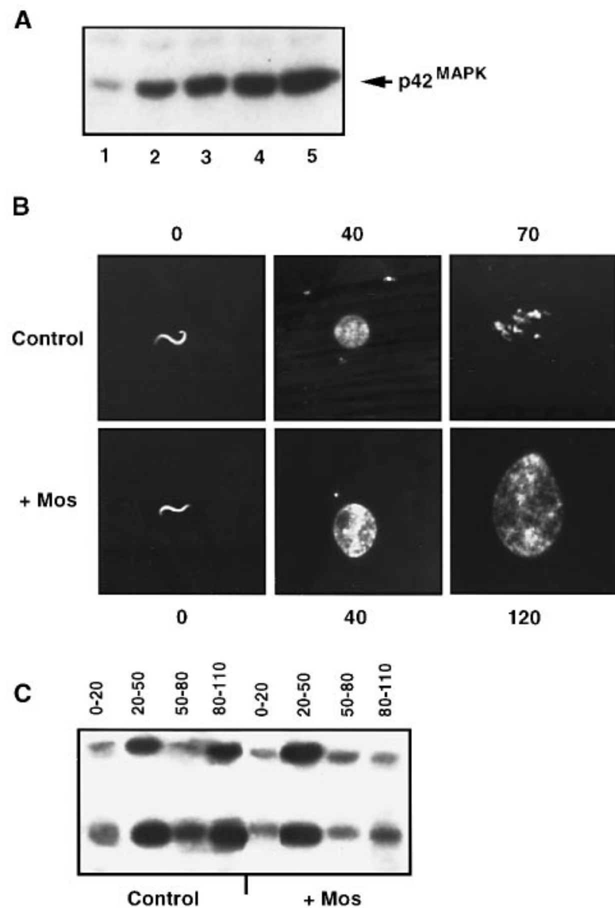


Fig. 1. Suppression of MAPK inactivation in *Xenopus* cell cycle extracts causes arrest of *in vitro* assembled sperm nuclei at G_2. (**A**) GST–Ste11–ΔN (lanes 2 and 3) or mal E-mos (lanes 4 and 5) fusion proteins were added or not (lane 1) soon after recovering, after centrifugation at 4°C, a cell cycle extract prepared from parthenogenetically-activated eggs, which was further incubated at 20°C. Samples containing identical amounts of egg extract were taken after 20 (lanes 1,2 and 4) and 60 min (lanes 3 and 5) and analysed for MAPK phosphorylation of MBP by $p42^{MAPK}$, using the in gel-MBP kinase assay. (B) and (C) Permeabilized sperm heads were added (500/μl) to a cell cycle extract prepared from *Xenopus* eggs containing (+Mos) or not (–, control) the recombinant proto-oncogene (similar results were obtained when GST–Ste11–ΔN was used instead of mal E-mos to suppress MAPK inactivation). (**B**) Samples were taken at the indicated times (in min) after sperm head addition and examined by Hoechst staining for assembly of sperm pronuclei and chromosome condensation. (**C**) [α-^{32}P]dCTP was added, either simultaneously with sperm heads (0), or 20, 50 or 80 min later. Samples were collected at 20, 50, 80 or 110 min after sperm head addition and incorporation of α^{32}P–dCTP in DNA was evaluated by gel electrophoresis and autoradiography in the indicated time windows.

Finally, we found that prevention of cyclin B–cdc2 kinase activation by clamping MAPK early in its active tyrosine-phosphorylated form was associated with accumulation in egg extracts of a tyrosine-phosphorylated 34 kDa protein (Figure 2E) identified as cdc2, as it could also be immunoprecipitated with specific anti-cdc2 antibodies (not shown). Accumulation of tyrosine-phosphorylated $p34^{cdc2}$ was not observed in extracts to

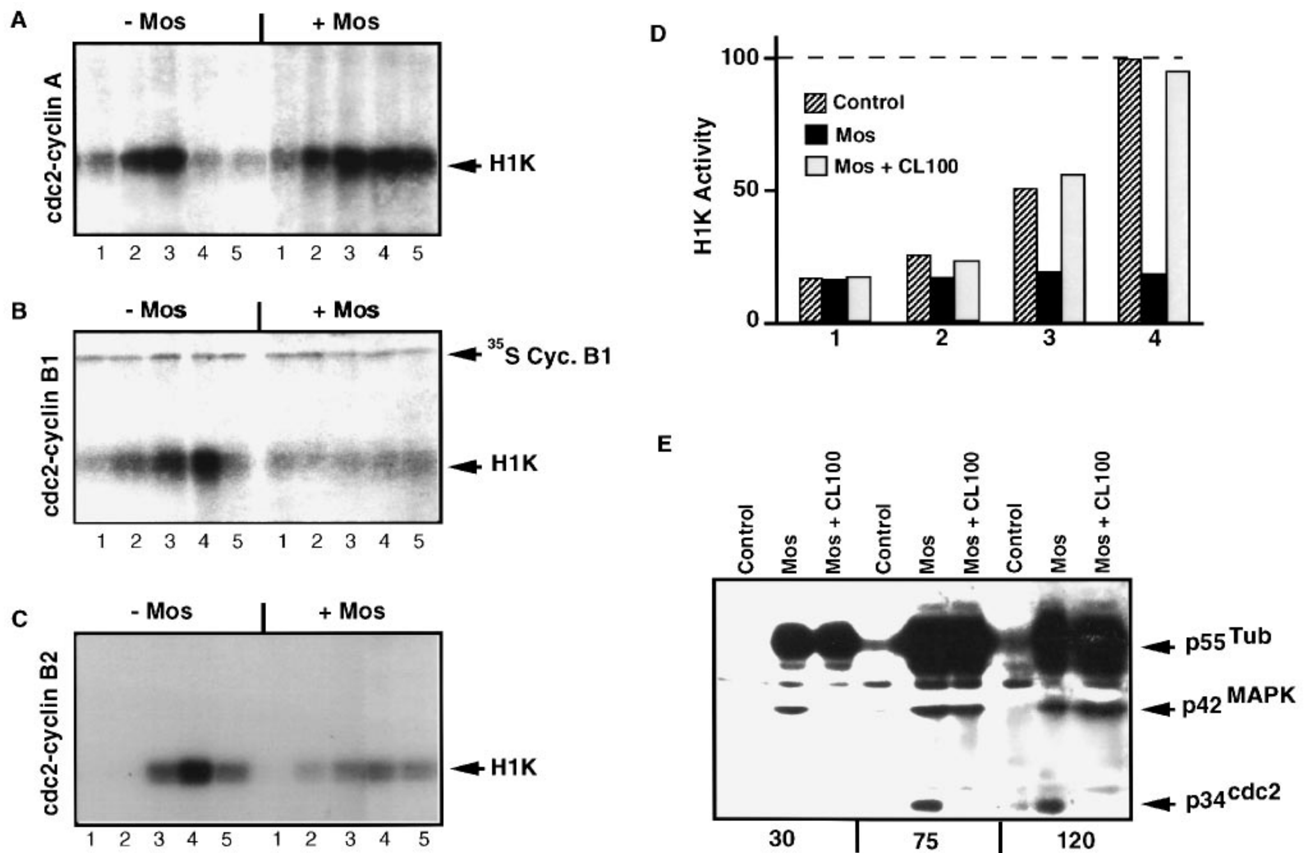


Fig. 2. Time-course of cdc2 kinase activities in cell cycle extracts prevented (+Mos) or not (–Mos) from inactivating MAPK by addition of the non-degradable Mos fusion protein. (**A**) H1 kinase activities were monitored by autoradiography after SDS–PAGE of anti-cyclin A immunoprecipitates prepared from aliquots taken after 20 (lane 1), 40 (lane 2), 50 (lane 3) 60 (lane 4) or 70 min (lane 5) of incubation at 20°C. (**B**) The same experiment, but H1 kinase activities were measured in anti-cyclin B1 immunoprecipitates. After diluting aliquots for immunoprecipitation in RIPA buffer, a tracer amount of ^{35}S-labelled cyclin B1 was added in each aliquot to evaluate immunoprecipitation recovery. ^{35}S-labelled cyclin B1 was also added before immunoprecipitation with anti-cyclin A in panel A experiments or anti-cyclin B2 in panel C experiments to control specificity of immunoprecipitation. (**C**) Same experiment, but H1 kinase activities were measured in anti-cyclin B2 immunoprecipitates. (**D**) Same experiment as in (B) (control, Mos), but H1 kinase activities of anti-cyclin B1 immunoprecipitates were quantified by liquid scintillation counting after SDS–PAGE, and are presented as percentages of control activity after 1 h (4). In parallel to these experiments, a sample of the same cell cycle extract received the CL100 recombinant protein, added simultaneously with Mos, at the time of transfer at 20°C. (**E**) The same experiment as in (D), but samples of egg extracts, taken 30, 75 and 120 min after transferring the cell cycle extract at 20°C, were analysed for immunoreactivity to anti-phosphotyrosine antibodies after SDS–PAGE and Western blotting. We previously showed that the prominent 55 kDa component recognized by our polyclonal anti-PTyr antibody in extracts containing the recombinant Mos protein is in fact tubulin, phosphorylated on serine residues exclusively (Abrieu *et al.*, 1996).

which both c-mos and CL100 were added simultaneously, even though they escaped from CL100 inhibition when incubated for more than 1 h at room temperature. This shows that MAPK no longer prevents newly synthesized cyclin B from forming active complexes with cdc2 once cdc2 kinase activation has started. Figure 2E further shows that CL100 suppressed MAPK, but not c-mos activity in egg extracts, as the tyrosine phosphatase did not prevent c-mos from phosphorylating a 55 kDa protein previously identified as tubulin (see Abrieu *et al*., 1996).

In the above experiments, recombinant mal E-mos (or GST–Ste11–ΔN) was added soon after centrifugation of homogenates at 4°C, so that MAPK targets were not expected to undergo dephosphorylation when the extracts were further incubated at 20°C. However, sperm nuclei did not systematically arrest at G_2, and were observed in some cases to rather arrest at mitosis with condensed chromosomes and high cdc2 kinase activity; this occurred when the recombinant MAPKKK was added 10 min or more after incubating extracts at 20°C, in agreement with previous reports (Abrieu *et al.*, 1996; Jones and Smythe, 1996). In this case, MAPK activity first dropped, then reappeared in egg extracts (not shown). We conclude that suppression of MAPK inactivation arrests cell cycle extracts at G_2, but reactivation of MAPK after its drop may arrest them either at G_2 or at metaphase.

Early inactivation of MAP kinase does not induce premature entry into first mitosis in Xenopus egg extracts

The above experiments suggested that MAP kinase may in some conditions act as a brake at the G_2 to M-phase transition. To investigate if MAP kinase actually plays this role in normal conditions, it was necessary to find a way to inactivate MAP kinase in advance of its normal schedule. In preliminary experiments, we failed to completely inactivate MAP kinase in CSF extracts using either the Pyst1 (Groom *et al.*, 1996) or the CL100 tyrosine phosphatases, unless MAP kinase was first partially depleted (about 50%, data not shown) from extracts using

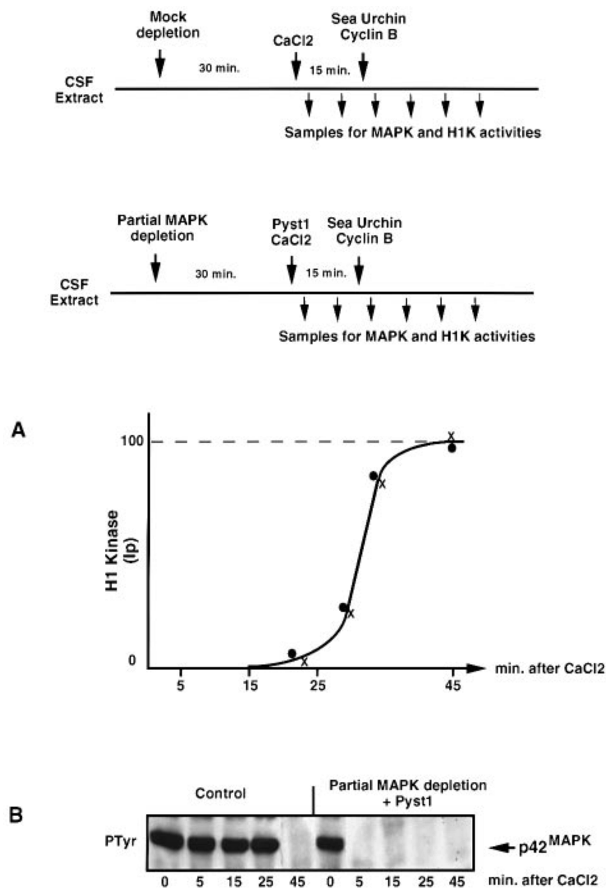


Fig. 3. Early inactivation of MAPK does not speed up cyclin B–cdc2 kinase activation in *Xenopus* egg extracts. Recombinant sea urchin cyclin B was added, 15 min after addition of 0.5 mM $CaCl_2$ to a CSF extract, in which early MAPK inactivation was induced (X) or not (l) through combined immunodepletion and Pyst–1 treatment. Samples were collected at the indicated times and analyzed for H1 kinase activities in anti-sea urchin cyclin B immunoprecipitates (**A**) or tyrosine phosphorylation of MAPK by immunoblotting (**B**).

specific antibodies. Using this specific double procedure, we first confirmed that inactivation of $p42^{MAP\ kinase}$ releases the cyclin degradation pathway from a CSF block in the absence of a Ca^{2+} transient (not shown). Minshull *et al.* previously reached the same conclusion using the CL100 tyrosine phosphatase, which could have inactivated other MAP kinases besides $p42^{MAP\ kinase}$ (Minshull *et al.*, 1994).

As even mock-immunodepletion abrogates spontaneous cell cycling in egg extracts (probably by depressing cyclin synthesis), the following procedure was designed to investigate the effect on the first mitotic cell cycle of prematurely inactivating MAP kinase (Figure 3). A CSF extract was divided into two parts. One was partially immunodepleted of MAPK content, the other mock-depleted. Thirty minutes later, both received 0.5 mM $CaCl_2$. In addition, the immunodepleted extract received recombinant Pyst1 protein. Fifteen minutes later, recombinant sea urchin cyclin B was added to both extracts, then samples were collected as a function of time and used for determination of H1 and MAP kinase activities.

No difference was detected in the timing of H1 kinase activation, as determined in immunoprecipitates using specific antibodies against sea urchin cyclin B (Figure 3A), even though tyrosine dephosphorylation and thus inactivation of MAPK occurred at least 20 min earlier in the Pyst1-treated extract (Figure 3B).

We conclude that early inactivation of MAP kinase does not speed up activation of cyclin B–cdc2 kinase or entry into mitosis when recombinant cyclin B is added to extracts first driven from metaphase to interphase by Ca^{2+} addition. This suggests that the time of MAP kinase inactivation may not be rate-limiting for the fertilized or parthenogenetically-activated *Xenopus* egg to progress into first mitosis. Unfortunately, we were unable to validate this conclusion in more physiological conditions, as the combined procedure we had to use to completely and reproducibly inactivate MAPK in advance of its normal schedule could not be applied to intact eggs or to cycling extracts.

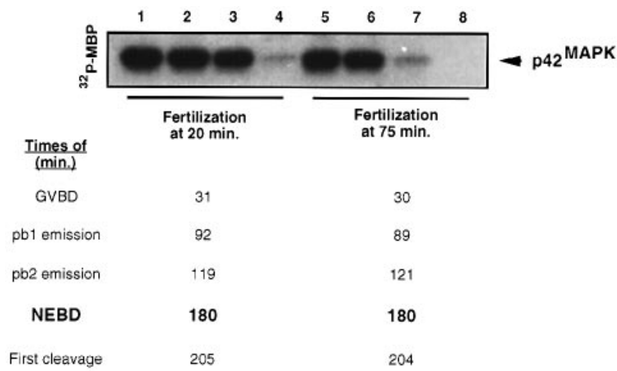
Thus we turned to another, more favourable biological system. We have previously reported that the length of the first mitotic cell cycle is longer than the following ones in the starfish *M.glacialis* (Picard *et al.*, 1988), and this holds true also for the starfish *Astropecten aranciacus* (not shown). We also found that MAPK activity drops at the time of second polar body emission in fertilized but not unfertilized eggs of *A.aranciacus*, and does not reappear later on (Picard *et al.*, 1996), at least during several cell cycles, as observed in *Xenopus*. However, an advantage of starfish, compared with most vertebrates, is that oocytes can be fertilized at various times during meiotic maturation (Cayer *et al.*, 1975; Schuetz, 1975; Peaucellier and Dorée, 1981). As we observed that the timing of MAPK inactivation somewhat depends on the time of fertilization, this provided us with the opportunity to examine in physiological conditions whether early inactivation of MAPK may reduce the length of the first mitotic cell cycle.

Early inactivation of MAPK does not reduce the length of the first mitotic cell cycle in starfish eggs

In the next experiment, oocytes of the starfish *A.aranciacus* were fertilized either 20 or 75 min after addition of 10^{-5} M 1-methyladenine, the natural inducer of meiotic maturation in starfish (Kanatani *et al.*, 1969). In both cases a single sperm penetrated each maturing oocyte, as the block to polyspermy is established shortly after hormone addition (Cayer *et al.*, 1975; Schuetz, 1975). The timing of maturation did not depend on the time of fertilization, and in both cases germinal vesicle breakdown (GVBD), first polar body emission and second polar body emission respectively occurred at the same time. In contrast, the time of MAPK inactivation was different (Figure 4). Whilst MAPK underwent inactivation later than 110 min post-hormone addition (p.h.a.) when oocytes were fertilized at 20 min p.h.a., MAPK inactivation occurred at least 10 min earlier when they were fertilized at 75 min p.h.a. In spite of this, the time of entry into the first mitosis (time of NEBD) and that of the first mitotic cleavage were identical in both groups of eggs. We conclude that the time of MAPK inactivation is not rate-limiting for progression of the first mitotic cell cycle in the intact starfish egg, as observed in the *Xenopus in vitro* assay.

Discussion

In the present study, we demonstrate that MAPK inactivation is essential for eggs, both vertebrate and invertebrate,



Times of (min.)	Fertilization at 20 min.	Fertilization at 75 min.
GVBD	31	30
pb1 emission	92	89
pb2 emission	119	121
NEBD	**180**	**180**
First cleavage	205	204

Fig. 4. The time of entry into first mitosis (NEBD, nuclear envelope breakdown) does not depend on the time of MAPK inactivation following fertilization. Prophase-blocked oocytes of the starfish *Astropecten aranciacus* were induced to mature with 0.1 µM 1 MeAde (time 0). Half of them were fertilized at 20 min post-hormone addition (p.h.a.) and the other half at 75 min p.h.a. At 80 min, 150 eggs of each batch were selected for a good fertilization membrane and homogenous 250 µm diameter. Upper autoradiogram: samples of 10 oocytes were taken at 85 min (lane 5), 95 min (lanes 1 and 6), 105 min (lanes 2 and 7), 115 min (lanes 3 and 8) and 125 min p.h.a. (lane 4), and processed for detection of in-gel MBP kinase activity. Lower table: each indicated cell cycle event was scored by examining samples every 5 min. The values are the interpolated times p.h.a. for 50% eggs having undergone each event in either batch.

to enter first mitosis. Down-regulation of MAPK is not required for Ca^{2+} to trigger degradation of mitotic cyclins at fertilization, and in fact it occurs much after eggs have exited meiotic metaphase (Lorca *et al.*, 1991, 1993; Watanabe *et al.*, 1991; Weber *et al.*, 1991). In contrast, suppressing down-regulation of MAPK, for example by microinjecting a constitutively active MAPKKK, suppresses entry into first mitosis. Conversely, inactivation of MAPK by the specific MAPK phosphatase Pyst1 is sufficient to drive the unfertilized mature egg into first mitosis.

Eggs of the starfish *A.aranciacus* and *M.glacialis*, as well as those of the amphibian *Xenopus*, do not require MAPK inactivation to replicate DNA in the first cell cycle. If prevented from inactivating MAPK, they replicate DNA, then arrest at G_2 with cyclin B–cdc2 kinase maintained in an inactive form, at least in part through phosphorylation of cdc2 on inhibitory residues. Besides this major effect, MAPK may slow down the first round of DNA replication. As a matter of fact, we have previously shown that the first round of DNA replication is completed much more rapidly (15 min instead of 45 min) in fertilized eggs of *M.glacialis*, which inactivate MAPK, than in fully-mature, unfertilized eggs which keep a high level of MAPK activity (see Figure 1 in Picard *et al.*, 1996). In the starfish *Asterina pectinifera*, MAPK has even been shown to completely block the first round of DNA replication in 70% of mature oocytes (Tachibana *et al.*, 1997). In this species, 30% of the eggs injected with constitutively active MAPKK escape G_1 arrest; in this case they arrest at G_2 after the first round of DNA replication (Tachibana *et al.*, 1997; K.Tachibana and T.Kishimoto, personal communication). It thus appears that the most general effect of maintaining MAPK activity, observed in all investigated species, vertebrate and invertebrate, is prevention of the G_2 to M-phase transition, in the first mitotic cell cycle at least. If eggs are first allowed to inactivate MAPK following fertilization, and MAPK is then inappropriately reactivated, the cell cycle may arrest at metaphase, at least in vertebrates (Sagata *et al.*, 1989; Haccard *et al.*, 1993; MacNicol *et al.*, 1995). This is, however, not observed in starfish and other invertebrates (A.Picard, unpublished results), that arrest at G_2 of the following cell cycle.

The mechanism responsible for MAPK-dependent inhibition of the G_2 to mitosis transition remains unknown. We show, using cycling *Xenopus* egg extracts, that MAPK specifically prevents cyclin B–cdc2 kinase activation, and has no effect on activation of cyclin A–cdc2. Moreover, prevention of cyclin B–cdc2 kinase activation by MAPK is associated with tyrosine phosphorylation of cdc2. However, a mutant of cdc2 that cannot be inhibited by phosphorylation has also been reported to be susceptible to inactivation in *Xenopus* egg extracts, demonstrating that inhibitory mechanisms independent of threonine–14 and tyrosine–15 phosphorylation may exist (Kumagai and Dunphy, 1995; Lee and Kirschner, 1996). Work is in progress to determine the respective contribution of this inhibitory pathway and of kinases that mediate the inhibitory phosphorylations on cdc2 in the MAPK-dependent G_2 to mitosis block.

A main objective of this work was to determine whether MAPK, active in the first but not the following cell cycles in early development of vertebrate and invertebrate embryos, acts as a brake in the fertilized or parthenogenetically-activated egg and is responsible for slowing down its progression into first mitosis. To address this question, we had to find a way to specifically inactivate MAPK in advance of its normal schedule, and investigate whether this would result in shortening of the first mitotic cell cycle. Microinjection of the MAPK-specific phosphatase Pyst1 was not sufficient in our hands to inactivate MAPK prematurely in parthenogenetically-activated *Xenopus* eggs or in extracts derived from them. However, the Pyst1 phosphatase readily inactivated MAPK prematurely in extracts undergoing parthenogenetic activation *in vitro*, provided they were partially immunodepleted of MAPK before parthenogenetic treatment. Using this dual procedure, we compared the time course of cyclin B–cdc2 kinase activation and onset of mitotic events following addition of recombinant cyclin B in *Xenopus* egg extracts undergoing early (~10 min) or late (~40 min) inactivation of MAPK following parthenogenetic activation. No difference was detected between 'early' and 'late' extracts.

These results argued against, but did not disprove, the hypothesis that MAPK might function as a brake on cell cycle progression during the first mitotic cell cycle. Indeed the dual procedure used for early MAPK inactivation could not be applied to intact *Xenopus* eggs. Fortunately, we were able to make complete the demonstration in physiological conditions, using starfish eggs which, like *Xenopus* eggs, have MAPK activity associated with the first mitotic cell cycle only, which proceeds more slowly than the following ones. At variance with *Xenopus* eggs, starfish eggs can be readily fertilized at various times during meiotic maturation. We found that the time of MAPK inactivation depends on the time when eggs are fertilized. However, the length of the first mitotic cell cycle did not depend on the time of MAPK inactivation. More specifically, advancing the time of MAPK inactiv-

ation did not speed up the schedule for entry of fertilized eggs into first mitosis. By inference, sustained activity of MAPK during part of the first mitotic cell cycle is not responsible for late entry of fertilized eggs into first mitosis, the reason for which remains unknown. The possibility that translation of mitotic cyclins or other regulators of mitotic progression might be rate-limiting during the first mitotic cell is unlikely, as suppressing protein synthesis after a 'point of no return' in the first S-phase hardly delays entry of both vertebrate and invertebrate eggs into first mitosis (Wagenaar, 1983; Solomon *et al.*, 1990; Genevière-Garrigues *et al.*, 1995).

The fact that (early) suppression of MAPK activity has no effect on cell cycle progression, whereas its prolongation or inappropriate reactivation arrests cell cycle progression at, possibly, different stages of the cell cycle, including entry into S-phase (*A.pectinifera)*, entry into mitosis (other starfish species and *Xenopus*) or exit from mitosis (*Xenopus*) is reminiscent of checkpoint mechanisms that operate to arrest or delay cell cycle progression if a defect that would compromise genetic stability is detected. It is already established that MAPK activity is required for the spindle assembly checkpoint that prevents cells whose spindles are defective or whose chromosomes are misaligned from initiating anaphase (reviewed by Minshull *et al.*, 1994; Murray, 1995; Takenaka *et al.*, 1997; Wang *et al.*, 1997). MAPK may act in this checkpoint by preventing MPF from turning on the cyclin degradation pathway *in vitro* (Abrieu *et al.*, 1996; Jones and Smythe, 1996; Takenaka *et al.*, 1997). Permanent MAPK activation mimics activation of the spindle assembly checkpoint and indeed arrests cell cycling *in vivo* (Takenaka *et al.*, 1997). The c-mos proto-oncogene, whose expression is limited to meiotic maturation of vertebrate oocytes, also arrests cell cycling at metaphase in unfertilized eggs through activation of the MAPK cascade (reviewed by Sagata, 1997; see also Furuno *et al.*, 1997). It would not be surprising that other checkpoints mechanisms use MAPK to arrest cell cycle at G_1/S or G_2/M in response to as yet uncharacterized deleterious stimuli. Inability of embryos to properly reactivate MAPK in many species during early cleavage could explain why they lack checkpoint mechanisms (Hartwell and Weinert, 1989; Clute and Masui, 1992; Murray, 1994; Clute and Masui, 1997) before a developmental transition in early development, which corresponds to MAPK reactivation. In agreement with this view, it was shown in *Caenorhabditis* that transgenic animals, expressing under control of a heat shock promoter a constitutively active MAPKK mutant with Ser223 mutated to Glu and Ser227 mutated to Asp, arrest eggs early in development when the heat shock was applied during early embryogenesis (Wu *et al.*, 1995).

Materials and methods

Xenopus egg extracts

Cycling and CSF-arrested extracts were prepared exactly as described in Morin *et al.* (1994), according to minor modifications of procedures described by Murray and Kirschner (1989). Procedures for assembly of sperm nuclei, cytological observations of nuclei and assay of DNA replication were as previously reported (Morin *et al.*, 1994).

Starfish oocytes

The starfish *A.aranciacus* was collected during its breeding season near the marine biological station of Banyuls. Fully grown oocytes (260 μm in diameter) were used throughout this work. Procedure for fertilization has been described previously (Picard *et al.*, 1988).

Recombinant proteins and mRNAs

The plasmids encoding mal E-mos, residues 370–717 of Ste11 in fusion with GST and the CL100 and Pyst1 MAPK phosphatases were generous gifts of Drs Hunt (London), Nishida (Kyoto) and Keyse (Dundee), respectively. Construction of the sea urchin GST–cyclin B has been described previously (Abrieu *et al.*, 1996). Recombinant proteins and mRNAs were prepared according to standard procedures.

Immunological procedures

The polyclonal antibody directed against phosphotyrosine has been described previously (Abrieu *et al.*, 1997). Polyclonal antibodies against *Xenopus* cyclin B1, cyclin B2 and cyclin A were raised by immunizing rabbits with the corresponding recombinant proteins. Immunoprecipitations were performed after dilution in RIPA buffer (Lorca *et al.*, 1992) and immunoprecipitates, collected on Protein A-Sepharose, were washed with 50 mM Tris (pH 7.5) before H1 kinase activities were assayed. Immunoblots were analysed by ECL.

Kinase assays

H1 kinase activities were assayed according to Labbé *et al.* (1991). Incubations were terminated by addition of Laemmli buffer, then proteins were separated by SDS–PAGE and phosphorylated H1 histone estimated either directly by liquid scintillation counting, or by autoradiography after Western blotting. In gel MBP-kinase activities were assayed exactly as described by Shibuya *et al. (*1992).

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