MAP kinase links the fertilization signal transduction pathway to the G₁/S-phase transition in starfish eggs

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The mechanism by which fertilization initiates S-phase in the zygote is examined by manipulating the activity of MAP kinase in mature starfish eggs. These unfertilized eggs, which are arrested at G₁-phase after the completion of meiosis, have high MAP kinase activity but undetectable cdc2 kinase activity. Either fertilization or inhibition of protein synthesis causes a decrease in MAP kinase activity, which is followed by DNA synthesis. Inactivation of MAP kinase with its specific phosphatase, CL100, initiates DNA synthesis in the absence of fertilization, while constitutive activation of MAP kinase with MEK represses the initiation of DNA synthesis following fertilization. Thus, in unfertilized mature starfish eggs, a capacity for DNA replication is already acquired, but entry into S-phase is negatively regulated by MAP kinase activity that is supported by a continuously synthesized protein(s) but not by cdc2 kinase. Upon fertilization, downregulation of MAP kinase activity is necessary and sufficient for triggering the G₁/S-phase transition.

Keywords: fertilization/G₁/S-phase transition/MAP kinase/mature starfish eggs/protein synthesis

Introduction

In the cell cycle of normal somatic cells, M-phase and S-phase alternate, the entry into M-phase being dependent upon the successful completion of S-phase and vice versa (Nurse, 1994). In contrast, during meiotic cycles there are two successive M-phases without an intervening S-phase; nevertheless, S-phase is initiated after the completion of meiosis when fertilization occurs (see Masui, 1985; Edgar and Lehner, 1996; Sagata, 1996). The molecular mechanism underlying the switchover of S-phase regulation through fertilization in oocytes and eggs is one of the most classic and essential problems to be resolved in the cell biology of early development.

Recent progress in studies of the cell cycle has established that the control machinery involved is composed of cyclin-dependent kinases (Cdks), cyclins and Cdk inhibitors which function effectively as an 'engine', an 'accelerator' and a 'brake', respectively (see Nigg, 1995; Labib and Moreno, 1996). Cell cycle progression is essentially regulated depending on the combination and balance of these elements. However, despite an understand-

ing of the roles of G₁-cyclins, their counterpart Cdks and Cdk inhibitors in the regulation of G₁-phase progression and S-phase onset in somatic cells (Sherr and Roberts, 1995), the molecular mechanism that links the fertilization stimulus to the initiation of S-phase remains obscure. A clue to the problem is provided by the indication of repression of DNA replication: entry into S-phase is suppressed in the interkinesis period during meiotic cycles of *Xenopus* oocytes in a protein synthesis-dependent manner (Ohsumi *et al.*, 1994) or by c-Mos (Furuno *et al.*, 1994; Sagata, 1996), and in *Drosophila* unfertilized eggs it is repressed by certain gene products of *plutonium* (*plu*), *pan gu* (*png*) or *giant nuclei* (*gnu*) (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991; Axton *et al.*, 1994).

In most animals, fully grown oocytes are arrested naturally at the first meiotic prophase before ovulation. On release from the ovary, oocyte maturation may resume and, depending on the species, fertilization occurs at a specific stage of oocyte maturation; the first meiotic prophase arrest, the first meiotic metaphase arrest, the second meiotic metaphase arrest, and the egg pronucleus stage after the completion of meiosis (Masui, 1985; Sagata, 1996). Accordingly, if fertilization occurs before the end of meiosis, the fertilization stimulus causes both the completion of the remainder of meiosis and subsequently, transition into S-phase. In contrast, when fertilization occurs at the egg pronucleus stage after meiosis has been completed, S-phase is initiated separately from the completion of meiosis, allowing us to examine the mechanism that couples fertilization stimulus with the initiation of S-phase in the absence of any effect of fertilization on the completion of meiosis. In this context, echinoderm oocytes complete meiosis in the absence of fertilization (Meijer and Guerrier, 1984; Kanatani, 1985; Kishimoto, 1996). These mature eggs are thought to be arrested at G₁-phase (pronuclear phase) unless fertilized, and fertilization causes the transition into S-phase. Here, we report that unfertilized mature starfish eggs, which are arrested at G₁-phase, have elevated levels of MAP kinase activity depending on protein synthesis, but not on cdc2 kinase, and that its inactivation is necessary and sufficient for triggering the G₁/S-phase transition at fertilization.

Results

MAP kinase-mediated G₁-phase arrest

Fertilization is known to initiate the first S-phase independently of new protein synthesis in echinoderm eggs, but the second S-phase is dependent upon completion of M-phase, which requires new protein synthesis (Wagenaar, 1983; Zhang and Ruderman, 1993). To confirm these previous reports, immature oocytes of the starfish, *Asterina pectinifera*, were treated with 1-methyladenine (1-MeAde)

Fig. 1. Unfertilized mature starfish eggs arrested at G_1 are already equipped with the machinery required for the entry into S-phase. (A–D) Unfertilized mature starfish eggs with a female pronucleus are arrested at G_1 . Immature oocytes of the starfish, *A. pectinifera*, were treated with 1-MeAde in the continuous presence of BrdU to undergo meiotic maturation. After the completion of meiosis II, mature eggs with female pronuclei were either uninseminated (A and B) or inseminated in the absence (C) or presence (D) of the protein synthesis inhibitor emetine, and subsequently incubated for 360 min (A and B) or 60 min (C and D). Note non-incorporation of BrdU into unfertilized female pronucleus (B), and its incorporation into nucleus after fertilization (C and D). Arrowhead in (A) indicates a female pronucleus. (E) The machinery for DNA replication is established during oocyte maturation. Immature starfish oocytes were treated with 1-MeAde in the continuous presence of BrdU and then inseminated just after germinal vesicle breakdown. Emetine was added to these oocytes at indicated times before or after 1-MeAde addition. Thereafter, BrdU incorporation was monitored after eggs were fixed at the first cleavage in control eggs which were untreated with emetine. Elevation of fertilization envelope was observed in every case in the presence of the protein synthesis inhibitor. In control oocytes untreated with emetine, after 1-MeAde addition germinal vesicle breakdown occurred at 20 min, extrusion of the first polar body at 60 min and the second polar body at 100 min, and formation of female pronucleus at 120 min (see also Figure 2A and C).

in the continuous presence of BrdU to complete meiotic maturation. After the female pronucleus was formed, mature eggs were inseminated in the presence or absence of the protein synthesis inhibitor, emetine (Figure 1A–D). No DNA synthesis was observed in unfertilized mature eggs, and fertilization initiated DNA synthesis regardless of the presence of the protein synthesis inhibitor (see also

Figure 5B). These observations indicate that unfertilized mature eggs of *A. pectinifera* are naturally arrested at G_1 after the completion of meiosis, and that in these eggs the components required for DNA replication are already present.

To address when the machinery required for DNA replication is established, immature oocytes were treated with 1-MeAde in the continuous presence of BrdU and then inseminated just after germinal vesicle breakdown, while emetine was added at various time points after 1-MeAde addition. When control eggs which were untreated with emetine underwent the first cleavage, emetine-treated eggs were fixed to monitor BrdU incorporation into the nuclei (Figure 1E). DNA synthesis was detectable in eggs to which the protein synthesis inhibitor had been added at meiosis II, but was undetectable when 1-MeAde was added at meiosis I. Thus, the ability to replicate DNA appears to be acquired during oocyte maturation, possibly by the end of meiosis I.

The fact that unfertilized mature starfish eggs arrested at G_1 are already equipped with the machinery for DNA replication raised the possibility that the initiation of DNA replication is repressed in these eggs. A candidate involved in such repression might be present in the MAP kinase pathway, since MAP kinase might act downstream (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993) of c-Mos to repress DNA replication in the meiotic interphase of *Xenopus* oocytes (Furuno *et al.*, 1994; Sagata, 1996). We therefore examined MAP kinase activity during starfish oocyte maturation and fertilization. As reported previously (Pelech *et al.*, 1988), MAP kinase was activated after cdc2 kinase activation and just after germinal vesicle breakdown. Thereafter, unless eggs were fertilized, elevated levels of MAP kinase activity were maintained even after the loss of cdc2 kinase activity at the completion of meiosis II (Figure 2A) as reported recently in oocytes of the starfish *Astropecten aranciatus* (Picard *et al.*, 1996). When mature eggs with female pronuclei were inseminated, fertilization triggered an immediate and rapid decrease in MAP kinase activity, which was followed by the initiation of DNA replication (Figure 2B). When insemination was performed during oocyte maturation, a similar immediate decrease of MAP kinase activity was observed in oocytes at meiosis II (Figure 2C). In oocytes at meiosis I, the decrease of MAP kinase activity occurred with a significant lag, that is, only after the completion of meiosis I, in spite of the immediate elevation of the fertilization envelope. It is likely that the machinery to decrease MAP kinase activity is established during meiosis I, while the fertilization stimulus for the MAP kinase inactivation appears to be retained during this period (see Nomura *et al.*, 1991). In any case, however, the initiation of DNA replication was observed at a definite time after the loss of MAP kinase activity (Figure 2C). All of these data indicate that the inactivation of MAP kinase is temporally followed by the entry into S-phase.

To address directly how MAP kinase is involved in the control of G_1 /S-phase transition, we have manipulated MAP kinase activity in mature starfish eggs by injecting mRNA for either its inactivator, CL100 (Keyse and Emslie, 1992; Alessi *et al.*, 1993), or its constitutively active activator, MEK^{DE} (Huang and Erikson, 1994). A MAP kinase-specific phosphatase, CL100, inactivated MAP

Fig. 2. MAP kinase inactivation correlates temporally with the entry into S-phase in starfish eggs. (A) Elevated levels of MAP kinase activity are maintained through oocyte maturation unless fertilized. Isolated immature starfish oocytes were treated with 1-MeAde to induce maturation, and MAP kinase activity was measured by MBP kinase assay at indicated times (■). Cdc2 kinase activity as represented by histone H1 kinase activity is shown as a meiotic cell cycle marker (○). (B) MAP kinase inactivation is followed by DNA replication after fertilization in mature eggs. Mature eggs were inseminated at the time indicated by an arrowhead, and DNA synthesis was monitored by the incorporation of BrdU (○). MAP kinase was monitored either by MBP kinase assay (lower panel; ■) or by immunoblots (upper panel); upper and lower bands (each indicated by arrowhead) correspond to the active and the inactive form of MAP kinase, respectively. (C) An ability for MAP kinase inactivation following insemination is acquired during oocyte maturation. Insemination was performed at indicated times (△) during oocyte maturation, and thereafter the changes in MAP kinase activity (■) and the initiation of BrdU incorporation (▶) were monitored. Levels of MAP kinase activity at the time of insemination are normalized to 100%. Each closed triangle at the bottom indicates the time of detectable incorporation of BrdU following the corresponding insemination indicated by each open triangle at the top (for example, a closed triangle with S30 indicates the time of S-phase initiation following insemination at an open triangle with i30, that is, at 30 min after 1-MeAde addition).

kinase significantly within 2 h in unfertilized mature eggs, and DNA synthesis was initiated in the absence of fertilization (Figure 3A). Injection of mRNA for a control, phosphatase-deficient mutant of CL100 had no effect. In contrast, mature eggs that had been previously injected with the constitutively active MAP kinase kinase, MEK^{DE}, failed to undergo DNA synthesis even after fertilization, while a control antisense mRNA of MEK^{DE} did not prevent the initiation of S-phase (Figure 3B). These observations indicate that inactivation of MAP kinase is necessary and sufficient to trigger the G₁/S-phase transition in mature starfish eggs.

Fig. 3. Regulation of MAP kinase activity is necessary and sufficient for the control of the G₁/S-phase transition in mature starfish eggs. (A) Inactivation of MAP kinase by CL100 initiates DNA synthesis in unfertilized mature eggs arrested at G₁. After injection of mRNA coding either wild-type or phosphatase-defective mutant CL100 into unfertilized mature eggs, DNA synthesis was examined by BrdU incorporation at 2 h (upper) and MAP kinase was monitored by immunoblots at 1 and 2 h (lower). Ten eggs were used for each lane. Compared with the mutant type, the wild-type CL100 appears to have significantly increased the number of BrdU-incorporated eggs, if the following points are taken into consideration; major inactivation of MAP kinase occurred on and after the first hour of the mRNA injection, and a small part of MAP kinase was still active at 2 h when the eggs were fixed for BrdU incorporation. It remains unclear, however, whether complete or partial DNA replication occurred in BrdU-incorporated eggs. (B) Maintenance of MAP kinase activity by constitutively active MEK^{DE} represses DNA synthesis following fertilization in mature eggs. Immature oocytes were injected with mRNA coding either constitutively active MEK or its antisense, and then treated with 1-MeAde in the presence of BrdU to undergo maturation. After the completion of meiosis II, mature eggs with a female pronucleus were inseminated, and 2 h later, DNA synthesis was examined by BrdU incorporation (upper) and MAP kinase was monitored by immunoblots (lower). In the absence of insemination, no BrdU incorporation was observed (see also Figure 1B). The fertilization envelope was observed to elevate normally in both cases of injection, but no cleavage was observed in sense MEK^{DE}-injected and inseminated eggs. Although it was practically difficult to monitor both BrdU incorporation and MAP kinase activity in the same egg, the ratio of eggs which incorporated BrdU was relatively high in a batch of experiments in which the injection with MEK^{DE} was less effective to support MAP kinase activity. This observation confirms that the ~30% of eggs which incorporated BrdU in spite of MEK^{DE} injection correspond mainly to those which failed to maintain MAP kinase activity at elevated levels rather than those which underwent DNA replication in spite of high level of MAP kinase activity. Alternatively, it may not be excluded that this 30% fraction of eggs underwent DNA replication but were arrested at G₂ in the presence of MAP kinase activity (see Discussion; Picard *et al.*, 1996). Since mRNA injection of MEK^{DE} appeared to be incompatible with the protein synthesis inhibition, the effect of MEK^{DE} on DNA synthesis initiated after protein synthesis inhibition was not examined (see Figure 5).

Protein synthesis-dependent suppression of S-phase initiation

In G₁-arrested mature starfish eggs, the data described earlier (Figure 2A) indicate the independence of MAP kinase activity on cdc2 kinase activity. To address how MAP kinase activity is supported in these eggs, we considered protein synthesis, as c-Mos, which might be located upstream of MAP kinase, is newly synthesized

Fig. 4. MAP kinase activity depends on protein synthesis in oocytes and mature eggs of starfish. (A) MAP kinase inactivation following protein synthesis inhibition in mature eggs. (B) Initial activation of MAP kinase at meiosis reinitiation is prevented by protein synthesis inhibition. Immature starfish oocytes were treated with 1-MeAde in the absence (A) or presence (B) of emetine. After the completion of meiosis II, emetine was added to mature eggs (A, arrowhead). MAP kinase activity (■) was monitored through these treatments. Cdc2 kinase activity (○) as represented by histone H1 kinase activity is shown as a meiotic cell cycle marker.

during *Xenopus* oocyte maturation (Sagata *et al.*, 1988). When mature eggs were treated with emetine, MAP kinase activity decreased rapidly (Figure 4A). A similar, albeit gradual, inactivation of MAP kinase was observed in mature eggs when puromycin was used as the protein synthesis inhibitor, while no changes were observed in protein levels of MAP kinase (see Figure 5A). At present, it is difficult to explain why puromycin required a much longer time to demonstrate any such effect than did emetine. Furthermore, although MAP kinase protein was already present in immature oocytes (data not shown), the initial activation of MAP kinase at meiosis reinitiation was completely prevented when emetine and 1-MeAde were added simultaneously to immature oocytes, while both the activation of cdc2 kinase and germinal vesicle breakdown occurred in meiosis I (Figure 4B). All of these data indicate the dependency of MAP kinase activity on protein synthesis. While basal levels of protein synthesis, though much lower than those observed after fertilization, were detectable in immature oocytes and unfertilized mature eggs (data not shown), MAP kinase activity appears to be supported by a continuously synthesized protein(s) which might be exhausted by turnover under the condition of protein synthesis inhibition.

When MAP kinase lost its activity following the addition of puromycin into unfertilized mature eggs (as expected from Figure 3A), DNA synthesis was initiated in the absence of fertilization (Figure 5A). A similar, but much earlier-appearing effect was observed when G_1 -arrested eggs were treated with emetine (data not shown; see Figure 5C). Following the incorporation of BrdU, an increase in the incorporation of [3 H]thymidine occurred ~4 h after addition of puromycin to unfertilized eggs, compared with such increase occurring only 30 min after

Fig. 5. Protein synthesis inhibition initiates DNA replication in unfertilized mature starfish eggs. (A) MAP kinase inactivation following protein synthesis inhibition correlates with the G_1 /S-phase transition in mature eggs. After the completion of meiosis II, unfertilized mature eggs were treated with puromycin. MAP kinase was monitored either by immunoblots (upper panel) or by MBP kinase activity (■). DNA synthesis was monitored by the incorporation of BrdU (○). (B) [3 H]Thymidine incorporation into puromycin-treated unfertilized eggs. After 30 min preincubation with [3 H]thymidine, unfertilized mature eggs were treated with puromycin, and processed for measurement of [3 H]thymidine incorporation at indicated times (□). As controls, [3 H]thymidine incorporation was measured in intact unfertilized eggs (■), fertilized eggs (●), and fertilized eggs followed by puromycin treatment (○). Abscissa indicates time after fertilization or puromycin addition. (C–E) BrdU incorporation by unfertilized mature eggs following treatment with protein synthesis inhibitors, and its inhibition by aphidicolin. Mature eggs that had completed meiosis II and formed an egg pronucleus were incubated for 360 min in the presence of emetine (C), puromycin (D), or both puromycin and aphidicolin (E). As a control, BrdU was not incorporated in untreated mature eggs (see Figure 1B).

fertilization (Figure 5B). The levels of [3 H]thymidine incorporation achieved over 6 h by eggs treated with puromycin reached at least 50% of those achieved during the first S-phase of fertilized eggs; that is, levels of incorporation seen in fertilized eggs in the presence of a protein synthesis inhibitor. The DNA synthesis seen in response to protein synthesis inhibition was blocked by aphidicolin (Figure 5C–E). These observations support the occurrence of a delayed, at least single round of DNA replication by the haploid genomes of unfertilized mature starfish eggs following inhibition of protein synthesis.

Discussion

We have demonstrated that, while mature eggs of the starfish *A.pectinifera*, are equipped with the machinery required for DNA replication, they are naturally arrested at G₁ in a MAP kinase-dependent manner. The machinery for MAP kinase inactivation is already established in mature eggs, whereas the MAP kinase activity is maintained by a protein synthesis-dependent process. In accordance with these findings, downregulation of MAP kinase activity is necessary and sufficient for entry into S-phase in mature eggs.

In budding yeast, the G₁ arrest induced by a mating pheromone is mediated by a member of the MAP kinase family, Fus3 (Peter *et al.*, 1993; Tyers and Fletcher, 1993; Peter and Herskowitz, 1994; Herskowitz, 1995). The substrate for Fus3 phosphorylation is Far1, and the phosphorylated Far1 associates with the Cln2–Cdc28 complex to inhibit its kinase activity, resulting in a cell cycle arrest at G₁. Upon passing through Start, Far1 degradation is suggested to release the inhibition from Cln2–Cdc28 kinase (McKinney *et al.*, 1993; Deshaies, 1995). This yeast pathway may be analogous to the role that we describe for starfish MAP kinase in ensuring G₁ arrest of mature eggs. Although it may be interesting to search for a starfish homologue of Far1, it remains unclear whether G₁-cyclin/Cdk complexes and protein degradation are required downstream of MAP kinase in order to initiate DNA replication after fertilization of starfish eggs. Rather, it is plausible that protein degradation following fertilization would be involved upstream of MAP kinase to suppress its activity, since both fertilization and inhibition of protein synthesis caused MAP kinase inactivation in mature eggs (see also below).

Recently, Picard *et al.* (1996) reported almost completely opposite results to those of the present study. In their starfish, *A.aranciacus* and *Marthasterias glacialis*, the cell cycle proceeds to and arrests at G₂ through S-phase after the completion of meiosis II in the absence of fertilization and in the presence of high MAP kinase activity. Furthermore, fertilization initiates S-phase, but G₂ arrest occurs in mature eggs if MAP kinase activity is supported by a constitutively active form of MAP kinase kinase from budding yeast, Δ N-STE11, whereas fertilization causes the inactivation of MAP kinase in untreated eggs. The discrepancy between these sets of results would possibly be interpreted by the absence or presence of a MAP kinase target that might be involved in the repression of the initiation of DNA replication. Although it is still unknown whether the MAP kinase target is related to Far1, its identification would possibly help to resolve the present confusion.

Alternatively, the MAP kinase-dependent block to DNA replication may be intermittent ('leaky'), depending on species. Indeed, even in *A.pectinifera*, control injection of phosphatase-deficient mutant of CL100 induced DNA synthesis in 15% of eggs, and fertilization induced DNA synthesis—but not first cleavage—in 30% of eggs injected with the constitutively-active MEK^{DE} (see also the legend to Figure 3). This 'leakiness' would be much more extensive in *A.aranciacus* and *M.glacialis*. Rather, in *A.pectinifera* eggs there may be an additional pathway that prevents the initiation of DNA replication independently of

MAP kinase activity. This notion is supported by the following observations. DNA replication in response to the injection of MAP kinase inactivator, CL100, was initiated in only 50% of the recipient population (Figure 3A), while DNA replication in response to protein synthesis inhibition was initiated when MAP kinase activity decreased to 50% of its starting level and ultimately occurred in almost 90% of eggs (Figure 5A). In addition, although more detailed analysis is required, re-replication might be induced by the protein synthesis inhibitor, as gradual and continuous incorporation of [³H]thymidine was observed in both fertilized and unfertilized eggs treated with the inhibitor (Figure 5B). Thus, it is likely that protein synthesis inhibition may have a positive effect on the process of DNA replication independently of its effect on the inactivation of MAP kinase. Taken together, for the repression of the initiation of DNA replication in G₁-arrested eggs, there may be two distinct pathways, both of which depend on protein synthesis, though one is mediated by MAP kinase and the other is not. This latter pathway may be lacking in some species of unfertilized mature eggs that are not arrested at G₁.

Various roles have been proposed for MAP kinase during oocyte maturation. First, in clam oocytes, a one-off transient activation of MAP kinase that is induced by fertilization releases the meiotic prophase arrest (Shibuya *et al.*, 1992). This requirement might be related to the role of MAP kinase for the maintenance of cdc2 kinase activity at the release from meiotic prophase arrest in *Xenopus* oocytes (Kosako *et al.*, 1994a; Nebreda *et al.*, 1995; Huang and Ferrell, 1996), and/or for the induction of early meiotic phenotypes (Choi *et al.*, 1996). Second, c-Mos, and hence, possibly its downstream effectors such as MAP kinase (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993), represses S-phase in the interkinesis period between meiosis I and II in *Xenopus* oocytes (Furuno *et al.*, 1994). Third, the Mos–MAP kinase pathway mediates the cytostatic factor (CSF)-induced meiotic metaphase II arrest in vertebrate oocytes through the maintenance of cdc2 kinase activity (Haccard *et al.*, 1993; Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Kosako *et al.*, 1994b; Sagata, 1996). Our present results with unfertilized starfish eggs suggest a fourth role of MAP kinase, namely the repression of the initiation of S-phase after completion of meiosis, ensuring G₁ arrest before fertilization. Finally, the fifth role is in the G₂ arrest of mature starfish eggs reported by Picard *et al.* (1996). It is plausible that these different roles of MAP kinase might depend on distinct types of MAP kinase-activating systems and MAP kinase targets (see Cano and Mahadevan, 1995; Herskowitz, 1995). In all roles except for the first, however, it might be deduced that MAP kinase in oocytes and eggs is involved in the prevention of parthenogenetic development, even if it functions at various stages of the cell cycle (Picard *et al.*, 1996; Sagata, 1996).

In view of the foregoing considerations, we propose a model for the control of G₁/S-phase transition at fertilization in starfish eggs (at least, in eggs arrested at G₁ unless fertilized) (Figure 6). Mature eggs acquire an ability to replicate DNA during oocyte maturation, but this ability is repressed by MAP kinase activity, which is supported by a continuously synthesized protein(s). When MAP

Fig. 6. A model for the initiation process of the first S-phase after fertilization in starfish eggs. A capacity for DNA replication is acquired during oocyte maturation, but in mature eggs it is repressed by the MAP kinase activity that depends on continuously synthesized protein(s), ensuring the G_1 arrest. At fertilization, MAP kinase is inactivated, and hence the repression is released, resulting in the transition from G_1 to S-phase. Protein synthesis may support the repression of DNA replication via another unknown pathway in addition to the MAP kinase-supporting system (see text for details).

kinase activity is elevated, the cell cycle of mature eggs remains arrested at G_1 . Fertilization induces the inactivation of MAP kinase activity, which in turn releases the repression of DNA replication. Thus, the transition from G_1 to S-phase following fertilization is executed by the removal of the MAP kinase-mediated repression of DNA replication. It is further possible that, in addition to MAP kinase activity, DNA replication is repressed in G_1 -arrested eggs by another pathway which depends on continuous protein synthesis and loses its ability following fertilization.

Materials and methods

Oocytes and eggs

Immature oocytes were isolated from the starfish, *A. pectinifera*, and treated with 1 μ M 1-methyladenine (1-MeAde) to induce maturation (Okumura *et al.*, 1996). In oocytes of this species, meiotic cell cycles proceeded after 1-MeAde addition typically as follows: germinal vesicle breakdown at 20 min, completion of meiosis I at 60 min, completion of meiosis II at 100 min and formation of female pronucleus at 120 min (see Figure 2A). Insemination was performed just after germinal vesicle breakdown or at the female pronucleus stage. In some experiments, oocytes and eggs were treated continuously with 0.1 mM emetine, 0.37 mM puromycin, or both 0.37 mM puromycin and 0.06 mM aphidicolin.

DNA synthesis assay

For labelling of individual nuclei, oocytes and eggs were incubated in the continuous presence of 10 mM bromo-deoxyuridine (BrdU) from 1-MeAde addition through fixation with methanol. After extraction with 4 M HCl, incorporation of BrdU was visualized as described by Nomura *et al.* (1991).

For measurement of [3 H]thymidine incorporation (Shimada and Terayama, 1976), mature eggs were incubated at 20°C under continuous agitation at a concentration of 10^4 eggs/ml in the continuous presence of 1 μ Ci/ml [3 H]thymidine (Amersham) dissolved in Jamarin U artificial seawater (Jamarin, Osaka) containing 0.05 mg/ml streptomycin and 50 U/ml penicillin-G. After 30 min preincubation, eggs were inseminated or added with puromycin at a final concentration of 0.37 mM. At indicated times, 0.5 ml of egg suspension was mixed with an equal volume of cold 20% TCA supplemented with 1 mg/ml cold thymidine. After centrifugation at 7500 g for 10 min, the pellet was washed seven times with 1 ml each of cold 5% TCA supplemented with 0.1 mg/ml thymidine, and finally dissolved with 100 ml of 0.5 M NaOH. Half of the solution was mixed with 1 ml of scintillation cocktail (ACSII, Amersham), and processed for liquid scintillation counting.

MAP kinase and histone H1 kinase assay

To each pack of oocytes or eggs was added 4 vols of an extraction buffer containing 160 mM Na- β -glycerophosphate, 40 mM EGTA,

30 mM MgCl₂, 200 mM KCl, 200 mM sucrose, 2 mM DTT, 1 mM Na-orthovanadate, 0.1% Nonidet P-40, 0.5 mM benzamide, 0.5 mM PMSF, 50 μ g/ml leupeptin and 50 μ g/ml soybean trypsin inhibitor, pH 7.3, and immediately frozen in liquid nitrogen. After thawing and sonication, oocyte or egg extracts were recovered following centrifugation at 12 000 g for 10 min. MAP kinase activity in the extracts was measured by in-gel assay of myelin basic protein (MBP) kinase activity as described by Gotoh *et al.* (1990), or monitored by immunoblots with anti-C-terminal peptide of rat ERK1 antibody (Seikagaku Kogyo, Tokyo). Histone H1 kinase activity in the extracts was measured as described (Ohsumi *et al.*, 1994). Both kinase activities were normalized so that in each of the figures, the rate of 100% corresponds to the highest level.

In vitro RNA synthesis and their injection

CL100 (Keyse and Emslie, 1992; Alessi *et al.*, 1993) and MEK1^{DE} (Huang and Erikson, 1994) mRNAs with 5'-cap were synthesized using mRNA Capping Kit (Stratagene) from 1 μ g of each linearized template DNA derived from cDNAs of CL100 and its phosphatase-deficient mutant inserted into pTZ vector, and cDNA of constitutively active MEK1^{DE} inserted into pGEM-7Zf(+) vector (gifts from Drs S.M.Keyse and N.Sagata, respectively). The mRNA was dissolved at 0.5 mg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and 200 pl each was injected as described (Kishimoto, 1986). Unfertilized mature eggs were injected with CL100 mRNA in the presence of BrdU. MEK1^{DE} mRNA was injected into immature oocytes, and within 2 h after the injection 1-MeAde was added to induce maturation. After the completion of meiosis II, these mature eggs containing MEK1^{DE} were inseminated in the presence of BrdU. Similar effects were observed when MEK1^{DE} mRNA was injected into mature eggs (data not shown).

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