MAP kinase does not inactivate, but rather prevents the cyclin degradation pathway from being turned on in *Xenopus* egg extracts

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**SUMMARY**

Unfertilized frog eggs arrest at the second meiotic metaphase, due to cytostatic activity of the c-mos proto-oncogene (CSF). MAP kinase has been proposed to mediate CSF activity in suppressing cyclin degradation. Using an in vitro assay to generate CSF activity, and recombinant CL100 phosphatase to inactivate MAP kinase, we confirm that the c-mos proto-oncogene blocks cyclin degradation through MAP kinase activation. We further show that for MAP kinase to suppress cyclin degradation, it must be activated before cyclin B-cdc2 kinase has effectively promoted cyclin degradation. Thus MAP kinase does not inactivate, but rather prevents the cyclin degradation pathway from being turned on. Using a constitutively active mutant of Ca²⁺/calmodulin dependent protein kinase II, which mediates the effects of Ca²⁺ at fertilization, we further show that the kinase can activate cyclin degradation in the presence of both MPF and the c-mos proto-oncogene without inactivating MAP kinase.

Key words: Cyclin degradation, MAP kinase, Calcium-calmodulin-dependent protein kinase-II, *Xenopus* egg

**INTRODUCTION**

In vertebrates, unfertilized eggs are arrested at the second meiotic metaphase, due to the activities of two dominant factors, MPF and CSF (Masui and Markert, 1971). MPF (M-phase promoting factor) was initially discovered as a factor that induces entry into metaphase when transferred into oocytes arrested at the G2/prophase boundary of the first meiotic cell cycle, and it was later shown to control entry into metaphase of eukaryotic cells universally (reviewed by Dorée, 1990 and Nurse, 1990). Conversely, MPF inactivation is required for eukaryotic cells to exit mitosis. Cytoplasm taken from unfertilized frog eggs also induces metaphase arrest and suppresses cleavage when transferred into one blastomere of a two-cell embryo, due to CSF (cytostatic factor). This suggests that frog eggs, and more generally vertebrate eggs, arrest at the second meiotic metaphase because they develop CSF activity during maturation, thereby preventing MPF inactivation and exit from M-phase.

MPF has been identified as an heterodimeric protein kinase composed of the cdc2 catalytic subunit and cyclin B as a regulatory subunit. Inactivation of MPF and exit from M-phase require ubiquitin-dependent proteolysis of the cyclin B subunit (Glotzer et al., 1991). Degradation of cyclin B only occurs slowly in cell-free extracts prepared at interphase, yet the rate of degradation increases considerably upon addition of purified cyclin B-cdc2 kinase (Félix et al., 1990; Lorca et al., 1992) or recombinant cyclin B, which forms an active complex with endogenous cdc2 in extracts.

The cyclin B-cdc2 kinase has therefore been proposed to promote degradation of mitotic cyclins by initiating a cascade of reactions including cyclin ubiquitinylation and ending with proteolysis, even though the mechanism by which cyclin B-cdc2 kinase regulates cyclin degradation remains almost completely unknown (for review, see Ciechanover and Schwartz, 1994).

Mature oocytes fail to arrest at the second meiotic metaphase when c-mos is disrupted by gene targeting (Colledge et al., 1994; Hashimoto et al., 1994). Conversely, microinjection of c-mos mRNA or protein into one blastomere of a two-cell frog embryo induces cleavage arrest at metaphase. These results demonstrate that c-mos is required for unfertilized vertebrate eggs to maintain the ubiquitin-dependent degradation of mitotic cyclins in a switched off state, even when cyclin B-cdc2 kinase activity is high.

MAP kinase (MAPK) has been proposed to mediate the CSF activity of c-mos, because the proto-oncogene actually induces MAPK activation in extracts of *Xenopus* oocytes or eggs (Nebrada and Hunt, 1993; Shibuya and Ruderman, 1993; Posada et al., 1993) through its phosphorylation. Moreover, microinjection of c-mos mRNA or protein into one blastomere of a two-cell frog embryo induces cleavage arrest at metaphase. These results demonstrate that c-mos is required for unfertilized vertebrate eggs to maintain the ubiquitin-dependent degradation of mitotic cyclins in a switched off state, even when cyclin B-cdc2 kinase activity is high.

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of c-mos is consistent with the fact that MAPK activation lags about 2 hours behind the onset of c-mos protein synthesis in maturing *Xenopus* oocytes, is inactivated approximately 20-30 minutes following fertilization, at the time of c-mos pro-telysis, and is not reactivated during early embryogenesis (Posada et al., 1991; Watanabe et al., 1991; Ferrell et al., 1991).

In the present work, we have developed an in vitro assay for CSF activity, as reconstituted in cell-free extracts prepared after parthenogenetic activation from *Xenopus* eggs. Using this assay, we have confirmed that MAPK blocks cyclin proteoly-sis if it is activated before MPF has turned on the ubiquitin-dependent degradation pathway. However MAPK has no such effect when it is activated after the degradation pathway has been turned on.

The multifunctional Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaM K II) has been shown to trigger Ca\(^{2+}\)-dependent inactivation of MPF and onset of anaphase through activation of the ubiquitin-dependent cyclin degradation pathway upon fertilization or parthenogenetic activation of frog eggs (Lorca et al., 1993; Morin et al. 1994). Using the same in vitro assay, we have further shown that MAPK inactivation is not required for CaM K II to turn on the cyclin degradation machinery in CSF extracts.

**MATERIALS AND METHODS**

**Egg extracts and spindle assembly**

Interphase egg extracts were prepared 40 minutes after partheno-genetic activation and assayed for cyclin degradation as previously described (Lorca et al., 1992). Cycling extracts were prepared essentially according to the method of Murray and Kirschner (1989), as described by Morin et al. (1994). To assemble spindles, demembranated frog sperm nuclei were added (200/μl) simultaneously with rhodamine-labeled tubulin (150 μg/ml), to cycling extracts. Spindles were observed with a fluorescent microscope, using Hoechst 33342 to stain chromosomes and tubulin fluorescence to visualize micro-tubules (Morin et al., 1994).

**Proteins**

Cyclin B-cdc2 kinase was prepared from maturing starfish oocytes at the first meiotic metaphase, as described elsewhere (Labbé et al., 1991). Recombinant sea urchin cyclin B-Δ90 and CL 100 proteins were produced in *Escherichia coli* and solubilized from inclusion bodies as previously described (Solomon et al., 1990; Keyse and Emrslie, 1992). The mal E-mos fusion protein was produced in *E. coli* and affinity-purified as described by Maina et al. (1988). Glutathione S-transferase-cyclin B (GST-cyclin B), which contains an N-terminal fusion of glutathione S-transferase (Smith and Johnson, 1988) to a sea urchin type B cyclin, was expressed in a *E. coli* strain that overexpressed the bacterial gene for the affinity tag. The mal E-mos fusion protein was added to a cycling extract prepared from parthenogenetically-activated eggs, and the mal E-mos fusion protein was added before the time of nuclear envelope breakdown. Whilst control untreated extracts exit from mitosis after chromatin segregation and enter the next cell cycle (not shown), cell cycle progression arrests at metaphase in extracts receiving the mal E-mos protein (Fig. 1) and sister chromatid segregation does not occur subsequently.

**Immunoblotting**

After SDS-PAGE, proteins were transferred to nitrocellulose membranes and probed with antibodies, using ECL detection (Amersham, International). Anti-phosphotyrosine polyclonal anti-

**Kinase assays**

Histone H1 kinases activities were assayed according to the method of Labbé et al. (1991). In the experiments depicted in Figs 3 and 6, MBP kinase activities were assayed by incubating oocyte extracts (15 μl) with a reticulocyte lysate (2 μl) programmed with HA-MAPK mRNA (Meloche et al., 1992). The tagged MAPK protein was recovered with the 12CA5 monoclonal antibody on Protein A-Sepharose beads that were washed with RIPA buffer (10 mM NaH\(_2\)PO\(_4\), pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 80 mM β-glycerophosphate, 50 mM NaF, 1 mM DTT), then with 50 mM Tris buffer, pH 8. The immune complexes were resuspended in 50 μl 50 mM Tris-HCl, pH 8, plus 10 μl of a cocktail containing 1 μCi [γ-\(^{32}\)P]ATP (3,000 Ci/mole Amersham), 10 μg myelin basic protein, 40 mM MgCl\(_2\); 80 mM Heps, 200 μM ATP. Reactions were performed at room temperature for 10 minutes and terminated by spotting 40 μl aliquots onto P81 phosphocellulose papers that were extensively washed with tap water before counting. In gel-MBP kinase assays were performed exactly as described by Shibuya et al. (1992).

**RESULTS**

An in vitro assay to generate CSF activity

Addition of the mal E-mos fusion protein to *Xenopus* egg extracts has been shown to reactivate MAP kinase (Nebrada and Hunt, 1993; Shibuya and Ruderman, 1993; Posada et al., 1993). However, it has not been documented whether mal E-mos was able to reconstitute CSF activity in such extracts. A characteristic property of genuine CSF is its ability to arrest progression of mitosis at metaphase when it is transferred into one of the two blastomeres of a two-cell embryo. To investi-gate if the mal E-mos protein could mimic genuine CSF and arrest cell cycle progression at metaphase, sperm nuclei were added to a cycling extract prepared from parthenogenetically-activated eggs, and the mal E-mos fusion protein was added before the time of nuclear envelope breakdown. Whilst control untreated extracts exit from mitosis after chromatin segrega-tion and enter the next cell cycle (not shown), cell cycle progression arrests at metaphase in extracts receiving the mal E-mos protein (Fig. 1) and sister chromatid segregation does not occur subsequently.

Another characteristic property of genuine CSF that defines its biological activity is suppression of ubiquitin-dependent degradation of mitotic cyclins. The effect of mal E-mos on the degradation of mitotic cyclins cannot be assayed directly in interphase extracts as these lack cyclin B-cdc2 kinase activity and therefore the cyclin degradation pathway is inactive. However, when the indestructable GST-cyclin B is added, to raise H1 kinase activity to about 10 picomoles/min/μl, the cyclin degradation pathway is turned on, as demonstrated by the rapid degradation of in vitro translated radiolabelled cyclin B1 (Fig. 2A).

In contrast, the cyclin degradation pathway is not turned on when the mal E-mos fusion protein is added before GST-cyclin B (Fig. 2B), even though GST-cyclin B does not generate a lower H1 kinase activity in the presence of mal E-mos (not
Recently, it was claimed that sea urchin cyclin B-D90 protein, which lacks the sequence required for ubiquitin-mediated degradation (Glotzer et al., 1991), causes degradation of endogenous cyclins in CSF extracts containing cycloheximide (Minshull et al., 1994). This was surprising, because addition of about 70 nM starfish cyclin B-D72 (which also lacks the degradation box) had not been found to induce degradation of full-length cyclins in CSF extracts in previous work, although it increased H1 kinase activity by about two-fold (Lorca et al., 1991). We therefore repeated the above experiments using the sea urchin cyclin B-D90 protein instead of cyclin B as an N-terminal fusion with GST. As shown in Fig. 2C, mal E-mos (20 ng/μl) still prevented the cyclin degradation from being turned on in such conditions. In the absence of mal E-mos, cyclin B-D90 readily turned on the cyclin degradation pathway (see below Fig. 7E).

In agreement with previous investigations, we confirmed that addition of mal E-mos to interphase extracts readily activates MAPK, as shown by its characteristic change in electrophoretic mobility on SDS-polyacrylamide gels (due to the phosphorylation of neighbouring threonine and tyrosine residues: Fig 3A), appearance of immunoreactivity to antiphos-
phototyrosine antibodies (Fig. 3B), and of MBP kinase activity in extracts (Fig. 3C).

In addition to MAPK, the polyclonal anti-P Tyr antibody was found to recognize a prominent 55 kDa component in extracts containing mal E-mos (Fig. 3B). When proteins were precipitated from extracts with 70% SO4(NH4)2 and resolubilized in the absence of ATP, immunoreactivity of the 55 kDa component decreased or disappeared, and it reappeared when ATP was added back. To confirm identification of phosphotyrosine in the 55 kDa protein, [\( ^{32}P \)]ATP was used in place of cold ATP. To our surprise, no phosphotyrosine was recovered after acidic hydrolysis. Rather, the 55 kDa protein was found to contain [\( ^{32}P \)]phosphoserine exclusively. Thus, for some unknown reason (perhaps proximity of an aromatic amino acid), the polyclonal antibody failed to discriminate the phosphorylated serine residue within the 55 kDa target from phototyrosine. As c-mos has been reported to associate with and phosphorylate tubulin (Zhou et al., 1991), we suspected the 55 kDa protein could be tubulin. In agreement with this, the 55 kDa protein was identified as tubulin by immuno-precipitation, and this was confirmed by western blotting after bidimensional analysis by gel electrophoresis of proteins in cell-free extracts (data not shown).

**Fig. 4.** The CL100 MAPK-inactivating phosphatase prevents mal E-mos from blocking cyclin degradation. Thirty minutes before GST-cyclin B, the proto-oncogene was added to interphase extracts either alone (1,2), or simultaneously with CL100 (3,4). Ninety minutes after the recombinant and non-degradable cyclin, full-length \(^{35}S\)-radiolabelled cyclin B1, translated in a reticulocyte lysate, was added. Samples were taken at 0 (1,3) or 60 minutes later (2,4) and analyzed either by fluorography for \(^{35}S\)-cyclin B1 degradation (\(^{35}S\)) or by western blotting with anti-phosphotyrosine antibodies (PTyr).

**c-mos-dependent activation of MAPK prevents MPF from inducing cyclin degradation, but does not turn off the cyclin degradation pathway**

Recombinant CL 100, a specific MAPK phosphatase that effectively inactivates MAPK in egg extracts, prevented mal E-mos from generating CSF activity, without suppressing tubulin phosphorylation (Fig. 4). This strongly suggests that the c-mos proto-oncogene blocks cyclin degradation through MAPK activation.

Yet, GST-cyclin B (Fig. 5) and cyclin B-\( \Delta 90 \) (Fig. 6A) were found to activate MAPK, even in the absence of mal E-mos, after a lag period the length of which varied from one experiment to another. Further addition of mal E-mos did not appear to increase MAPK activity, as judged from anti-PTyr immunoreactivity. This was confirmed using either an in gel-MBP kinase assay (Fig. 5) or a tagged HA-MAPK translated in a reticulocyte lysate and transferred into interphase extracts (Fig. 6B). Thus, although GST-cyclin B and mal E-mos activate MAPK in interphase extracts, those containing only GST-cyclin B readily degrade, whilst those first receiving mal E-mos do not degrade, mitotic cyclins.

The above results supported the view that c-mos could suppress cyclin degradation only if MAPK activation occurred before cyclin B-cdc2 kinase activation. To investigate this possibility, we took advantage of the fact that non-degradable cyclins do not merely turn on, but also normally maintain the cyclin degradation pathway in a turned on state when added to interphase *Xenopus* extracts, in contrast to degradable cyclins, or purified MPF (Fig. 7A). It takes about 20 minutes for bacterially-produced mal E-mos to trigger MAPK activation in interphase extracts (not shown; see Nebrada and Hunt, 1993). When added 30 minutes or more before cyclin B-\( \Delta 90 \), the proto-oncogene protein readily suppressed cyclin degradation (Fig. 7B). In contrast, it failed to suppress cyclin degradation when added after (Fig. 7C), or even simultaneously (Fig. 7D) with cyclin B-\( \Delta 90 \), although MAPK was maximally activated in these experiments at the time when extracts were assayed for cyclin degradation by adding \(^{35}S\)-labelled cyclin B1.

We previously showed that okadaic acid and microcystin, two protein phosphatase inhibitors, prevent inactivation of the
ubiquitin-dependent degradation of mitotic cyclins in *Xenopus* egg extracts (Lorca et al., 1991) and this observation was recently used to isolate a constitutively active ‘cyclosome’ from clam oocyte extracts (Sudakin et al., 1995). As shown in Fig. 8, microcystin not only induced constitutive activation of the cyclin degradation pathway, but also MAPK activation. This confirms that active MAPK does not necessarily give rise to a block of cyclin degradation.

**CaM KII releases the cyclin degradation pathway from the CSF block without inactivating MAPK**

We previously showed that, even in the absence of Ca\(^{2+}\), addition of a constitutively active CaM KII mutant to a cell-free extract prepared from metaphase II-arrested *Xenopus* eggs triggers cyclin degradation and onset of anaphase (Lorca et al., 1993; Morin et al., 1994). Although unlikely, from the work of Nebraska and Hunt (1993), we considered the possibility that MAPK inactivation could be required for CaM KII to release cell-free extracts from CSF block and induce cyclin degradation.

Therefore, CSF activity was generated in vitro (as ascertained in Fig. 9A) and CaM KII added to the arrested extract (Fig. 9B). As expected, this readily activated the cyclin degradation pathway (Fig. 9B). Yet MAPK did not undergo tyrosine dephosphorylation, and tubulin did not undergo dephosphorylation either. Similar results were obtained when 0.4 mM CaCl\(_2\) was added to the cell-free extract instead of CaM KII to turn on the cyclin degradation pathway (not shown).
Although the above results strongly suggested that MAPK inactivation is not required for CaMKII to turn on the cyclin degradation pathway, consistent with the reported chronology of cyclin degradation and MAPK inactivation following fertilization, a Ca\(^{2+}\) pulse or the constitutively active CaMKII could conceivably cause a transient down regulation of MAPK. This could be sufficient to create a window in which cyclin degradation could be turned on, followed by resumption of MAPK activity, which would then no longer be cytostatic. In order to exclude such a possibility, we carried out a more detailed examination of MAPK activity after introduction of CaMKII. Fig. 9C shows the typical results of such an experiment, in which MAPK activities of samples, taken as a function of time after CaMKII addition, were quantified by in-gel MBP phosphorylation for MAPK activity (C).

DISCUSSION

Much evidence supports the view that the c-mos protooncogene stabilizes MPF through activation of MAPK, but how MAPK acts to suppress cyclin degradation has not been elucidated, perhaps because of the lack of a functional in vitro assay. A first attempt to maintain MPF activity by adding a c-mos recombinant protein to cell-free extracts may have failed because oocytes, from which extracts had been prepared, lacked a component required, besides MAPK, to block cyclin degradation (Nebrada and Hunt, 1993). More recently, however, it was shown that recombinant c-mos prevents cycling and maintains H1 kinase activity at a high level in extracts prepared from parthenogenetically activated \textit{Xenopus} eggs (that lack CSF), and that further addition of a MAPK-inactivating phosphatase restores H1 kinase inactivation, consistent with the view that MAPK is required to suppress cyclin degradation (Minshull et al., 1994).

In the present work, we have shown that addition of recombinant c-mos protein to a non-cycling extract prepared from parthenogenetically-activated \textit{Xenopus} eggs prevents further addition of cyclin B, or cyclin B-cdc2 kinase, from turning on the cyclin degradation machinery. We confirmed that c-mos blocks cyclin degradation through MAPK activation by showing: first, that the CL100 MAPK phosphatase, which effectively dephosphorylates MAPK in extracts, restores cyclin degradation; and second, that it does not suppress the ability of c-mos to phosphorylate tubulin in the same extracts. The importance of this control should be stressed, in view of...
that the complexity of feedback loops in the MAPK cascade (for reviews, see Kosako et al., 1994; Herskowitz, 1995).

Using this simple in vitro assay, we addressed the question as to whether MAPK inactivates the cyclin degradation pathway, as it seems to have been largely assumed previously (Minshull et al., 1994; Kosako et al., 1994; Haccard et al., 1993), or if it rather prevents cyclin B-cdc2 kinase from promoting cyclin degradation. It should be stressed that this question was addressed in the absence of added microtubule depolymerizing drugs, and in conditions where no further checkpoint mechanisms are believed to be activated.

We found that for MAPK to suppress cyclin degradation, it must be activated before cyclin B-cdc2 kinase has effectively promoted cyclin degradation. This may explain why cells that contain both active MPF and MAPK do not necessarily arrest at metaphase, as observed in at least some mammalian cells in culture (Tamemoto et al., 1992), or in oocytes of many species, either vertebrate or invertebrate (Sanghera et al., 1990; Shibuya et al., 1992; Jessus et al., 1991; A. Picard, unpublished results).

In at least some of them, including *Xenopus* and starfish, cyclin degradation is even advanced, and already occurs at prometaphase (Galas et al., 1993; Furuno et al., 1994), even though MAPK is already maximally activated at that time. Conversely, our results are consistent with the fact that c-mos causes arrest of vertebrate oocytes at the second meiotic metaphase. Indeed MAPK activity does not drop between the first and the second metaphase, in contrast to MPF, thus MAPK is activated well before MPF during the second meiotic cell cycle.

At fertilization, an increase in the cytoplasmic calcium concentration acts through CaM KII to trigger cyclin B destruction and inactivation of MPF (Lorca et al., 1993). Using a constitutively active CaM KII mutant, we now present evidence that CaM KII releases the pathway leading to cyclin degradation from MAPK inhibition without inactivating MAPK. This is consistent with the fact that MAPK inactivation does not occur earlier than 20-30 minutes after fertilization, which is well after cyclin degradation. We postulate that a substrate of MAPK (X) inhibits some step in the cascade leading from MPF activation to cyclin ubiquitylation and degradation. As CaM KII releases this block without inactivating MAPK, it may either activate a phosphatase responsible for dephosphorylation of inhibitor X or act downstream of inhibitor X to suppress its cytostatic effect. A few proteins have been shown to undergo phosphorylation following fertilization (Caponi et al., 1986); one of them could be a substrate of CaM KII that mediates its effect on CSF activity.

In the mouse oocyte, arrest at metaphase is also due to c-mos and thus is mediated by MAPK (Colledge et al., 1994; Hashimoto et al., 1994). Nocodazole treatment prevents oocytes from completing metaphase following parthenogenetic activation with the Ca2+ ionophore A 23187 (Kubiak et al., 1993; Winston et al., 1995), suggesting that a Ca2+-dependent enzyme, presumably CaM KII, requires microtubules in this system to release oocytes from MAPK-dependent inhibition of cyclin degradation. Simultaneous treatment with dimethyl-adenine, a protein kinase inhibitor with preference for proline-directed kinases (M. Basset and M. Dorée, unpublished results) releases oocytes from metaphase arrest, even in the presence of nocodazole (Moses et al., 1995), although dimethyladenine alone has no effect. This suggests that the pathway leading to activation of the cyclin degradation machinery may not only depend on MAPK and CaM KII activity, but also on dynamics of the microtubule network.

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