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The c-*mos* proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs

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During studies of the activation and inactivation of the cyclin B–p34^{cdc2} protein kinase (MPF) in cell-free extracts of *Xenopus* oocytes and eggs, we found that a bacterially expressed fusion protein between the *Escherichia coli* maltose-binding protein and the *Xenopus* c-*mos* protein kinase (*malE–mos*) activated a 42 kDa MAP kinase. The activation of MAP kinase on addition of *malE–mos* was consistent, whereas the activation of MPF was variable and failed to occur in some oocyte extracts in which cyclin A or okadaic acid activated both MPF and MAP kinase. In cases when MPF activation was transient, MAP kinase activity declined after MPF activity was lost, and MAP kinase, but not MPF, could be maintained at a high level by the presence of *malE–mos*. When intact oocytes were treated with progesterone, however, the activation of MPF and MAP kinase occurred simultaneously, in contrast to the behaviour of extracts. These observations suggest that one role of c-*mos* may be to maintain high MAP kinase activity in meiosis. They also imply that the activation of MPF and MAP kinase *in vivo* are synchronous events that normally rely on an agent that has still to be identified.

Key words: cell cycle/maturation/meiosis/oncogene/signal transduction

Introduction

Fully grown *Xenopus laevis* oocytes are arrested at the G_2/M border of first meiotic prophase. Progesterone releases this arrest by activating a number of protein kinases that initiate germinal vesicle breakdown (GVBD) and meiotic maturation, leading to the production of the unfertilized egg, which is arrested at metaphase of meiosis II (Kirschner *et al.*, 1985; Cicirelli *et al.*, 1988; Maller, 1990). The most important event in this process is thought to be the activation of cyclin B–p34^{cdc2} kinase (MPF), which is stored in *Xenopus* oocytes as an inactive complex called pre-MPF (Cyert and Kirschner, 1988; Gautier and Maller, 1991). The activation of pre-MPF requires a tyrosine-specific phosphatase encoded by *cdc25*, which dephosphorylates p34^{cdc2} at Tyr15 and possibly also Thr14 (Gould and Nurse, 1989; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991, 1992; Millar and Russell, 1992). The activity of *Xenopus* cdc25 can be increased by protein phosphorylation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), but it is not known which protein kinase is responsible for this during oocyte maturation, or indeed whether activation of cdc25 is the sole key to turning on MPF. Potential candidates include S6 kinase, c-*mos* and MAP kinase, which are sequentially activated before or at about the same time as MPF during the course of maturation of *Xenopus* oocytes (Cicirelli *et al.*, 1988; Sagata *et al.*, 1988, 1989a; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a,b; Posada *et al.*, 1991; Barrett *et al.*, 1992; Lane *et al.*, 1992). MAP kinase(s) have been identified in many cell types and are activated by extracellular stimuli via a protein kinase cascade (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Alessandrini *et al.*, 1992; Kosako *et al.*, 1992; Kyriakis *et al.*, 1992; Nakielny *et al.*, 1992; Posada and Cooper, 1992). It has been reported that purified MPF can activate MAP kinase in *Xenopus* oocyte extracts (Gotoh *et al.*, 1991a), although in clam oocytes MAP kinase is activated shortly before the major burst of MPF activity (Shibuya *et al.*, 1992a). Recently, Shibuya *et al.* (1992b) showed that oncogenic *ras* protein led to the activation of MAP kinase in *Xenopus* extracts, but no activation of MPF occurred, even though oncogenic *ras* can promote maturation in intact oocytes (Birchmeier *et al.*, 1985).

Progesterone-induced meiotic maturation requires ongoing protein synthesis, a point that has been the subject of intense studies in recent years. Several mRNAs start to be translated in response to progesterone, including cyclins A and B1, p33^{cdk2} and the product of the c-*mos* proto-oncogene, a 39 kDa serine/threonine protein kinase (Sagata *et al.*, 1988, 1989a; Freeman *et al.*, 1990; Kobayashi *et al.*, 1991; Paris *et al.*, 1991). Antisense ablation of c-*mos* mRNA blocks oocyte maturation (Sagata *et al.*, 1988), whereas ablation of cyclin mRNA does not (Minshull *et al.*, 1991), implying that synthesis of c-*mos* is a necessary component of the maturation pathway(s) that triggers the activation of MPF. Moreover, bacterially expressed c-*mos* protein can promote oocyte maturation even in the absence of protein synthesis (Yew *et al.*, 1992), and the protein kinase inactive forms of c-*mos* are ineffective (Freeman *et al.*, 1989; Yew *et al.*, 1992); however, the salient substrates of c-*mos* during oocyte maturation are not well characterized. The c-*mos* protein kinase can associate with and phosphorylate α- and β-tubulin (Zhou *et al.*, 1991), but the significance of this property for the induction of maturation is not clear. A suggestion that B-type cyclins were targets for the c-*mos* protein kinase (Roy *et al.*, 1990), implying the possibility of a direct role of c-*mos* in the activation and stabilization of MPF, has recently been questioned (Xu *et al.*, 1992).

We have been testing the ability of a number of known activators of maturation in intact *Xenopus* oocytes for their ability to activate MPF in concentrated cell-free extracts prepared from resting oocytes. In this paper, we show that addition of recombinant c-*mos* protein to such extracts induces the rapid activation of MAP kinase, but not (or at least not reliably) MPF. The same activation occurs in interphase extracts of *Xenopus* eggs, indicating that this mode of MAP kinase activation is not restricted to oocytes. We

discuss the implication of these findings for the pathways of oocyte activation.

Results

malE−mos activates a myelin basic protein kinase in extracts of Xenopus oocytes

To study the biochemical events that underlie the activity of c-*mos* in *Xenopus* oocytes, we prepared concentrated cell-free extracts from collagenase-treated oocytes (see Materials and methods) following protocols based closely on those pioneered by Lohka and Masui (1984) and refined by Murray (1991) for *Xenopus* eggs. Since recombinant *malE−mos* protein (*Xenopus* c-*mos* fused downstream of the maltose-binding protein of *Escherichia coli*) can promote oocyte maturation in the absence of protein synthesis (Yew *et al.*, 1992), we tested whether it would also activate protein kinases in these cell-free extracts. After addition of buffer or recombinant *malE−mos*, samples were incubated at 23°C and assayed at intervals for their histone H1 kinase activity. Figure 1 shows that unstimulated oocytes contained very low histone H1 kinase activity, which underwent a large increase after exposure to progesterone, concomitant with the activation of cyclin B−p34^{cdc2} kinase (Figure 1A, lanes 1 and 2). Addition of recombinant *malE−mos* protein did not significantly activate histone H1 kinase (Figure 1A, lanes 3−6). However, we noticed a reproducible increase in the phosphorylation of endogenous substrates when *malE−mos* was added, indicated by small arrows in Figure 1A. These bands could either represent substrates for the added *malE−mos* kinase or reflect the activation of other protein kinase(s) present in these extracts as inactive precursor(s), or both. Since MAP kinase is known to be activated during oocyte maturation, we tested the ability of the extracts incubated with *malE−mos* to phosphorylate myelin basic protein (MBP), a well characterized *in vitro* substrate of MAP kinases (Erickson *et al.*, 1990). Figure 1B shows that MBP kinase activity increased 9-fold after addition of *malE−mos* to the oocyte extracts. There was a lag of ~10 min before any increase in MBP kinase activity occurred in the *malE−mos*-treated extracts, which probably represents the time taken for activation of the recombinant *malE−mos* protein in the extracts, as well as for the activation of the downstream kinase(s). The rate of activation of MBP kinase was a function of the concentration of *malE−mos* used; as little as 3 μg/ml (37 nM final concentration in the extracts; for comparison, p34^{cdc2} is present at ~800 nM) resulted in a slow but significant increase in MBP kinase activity (Figure 1B). No effect was observed when the *malE−mos* protein preparations were boiled before addition to the extracts, and the *malE−mos* preparations themselves had no detectable kinase activity when assayed with histone H1 or MBP as substrate. No increase in MBP kinase activity was detected after addition of purified maltose-binding protein, human protein kinase A inhibitor or *Xenopus* MAP kinase fused to the maltose-binding protein of *E.coli* and purified in the same way as the *malE−mos* protein (data not shown). Protein synthesis in the oocyte extracts was not required for the activation of MBP kinase by *malE−mos*, since the process occurred with similar kinetics in the presence of 100 μg/ml cycloheximide.

The malE−mos activated MBP kinase is MAP kinase

The most likely identity of the MBP kinase activated by *malE−mos* is the 42 kDa MAP kinase that has been extensively studied by several groups in *Xenopus* (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a,b; Posada *et al.*, 1991). Activation of MAP kinases requires phosphorylation on neighbouring threonine and tyrosine residues catalysed by MAP kinase kinase (Gomez and Cohen, 1991; Howe *et al.*, 1992; Posada and Cooper, 1992), reviewed by Sturgill and Wu (1991). This phosphorylation causes a change in electrophoretic mobility on SDS−polyacrylamide gels that is seen during the normal meiotic maturation of *Xenopus*

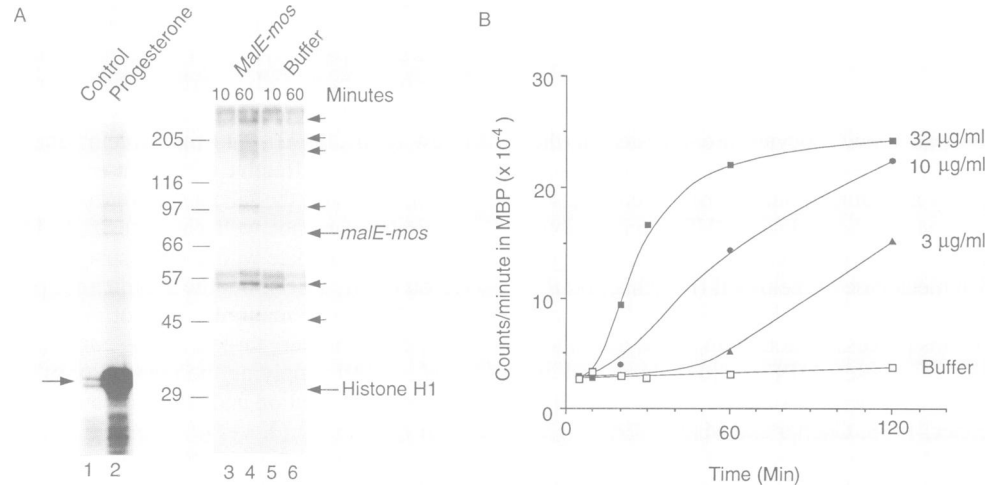

Fig. 1. Recombinant *malE−mos* protein activates protein kinase in *Xenopus* oocyte cell-free extracts. **A.** The H1 kinase activity of cell-free extracts incubated with 32 μg/ml of *malE−mos* or an equivalent volume of buffer, as indicated. As a control, lysates prepared from oocytes unstimulated (lane 1) or matured with progesterone for 15 h (lane 2) were tested. The positions of the *malE−mos* fusion protein and histone H1 are shown. Small arrows indicate the increased phosphorylation of endogenous substrates in *malE−mos*-incubated extracts. **B.** The time-course of activation of MBP kinase by *malE−mos*. Cell-free extracts were incubated with buffer or the indicated amounts of *malE−mos* and then assayed in duplicate for MBP kinase activity. Background phosphorylation in the absence of exogenous MBP was 9800 c.p.m. with buffer and 12 600 c.p.m. with 32 μg/ml of *malE−mos*. We estimated that after 60 min of incubation with 32 μg/ml of *malE−mos*, the oocyte extracts were able to incorporate ~1700 c.p.m. (0.34 pmol phosphate) in MBP/min/μg of total protein in the extract.

oocytes (Gotoh *et al.*, 1991a; Posada *et al.*, 1991) (Figure 2B, lanes 9–11). To confirm that the increased activity in MBP kinase of the *malE–mos*-treated oocyte extracts (Figure 2A, lanes 7 and 8) was due to activation of MAP kinase, we probed immunoblots of the extracts with an anti-MAP kinase monoclonal antibody that recognizes the purified *Xenopus* MAP kinase. A shift in electrophoretic mobility of the endogenous MAP kinase occurred in parallel with the appearance of MBP kinase activity (Figure 2B, lanes 5–8). When these immunoblots were probed with an anti-phosphotyrosine antibody, the upper band was strongly stained, while the lower band was unreactive (not shown). The MBP kinase activity of oocyte extracts incubated with *malE–mos* co-eluted with the endogenous MAP kinase upon gel filtration chromatography (data not shown).

Finally, we used an anti-*Xenopus* MAP kinase kinase antiserum (Kosako *et al.*, 1992) to prepare immunoprecipitates from oocyte extract that had been incubated with *malE–mos*. A doublet of ~45 kDa, the expected size of *Xenopus* MAP kinase kinase (Kosako *et al.*, 1992), became significantly labelled (probably by autophosphorylation) upon incubation of the immunoprecipitates with [γ-^{32}P]ATP (Figure 3). This result suggests that activation of MAP kinase by *malE–mos* in cell-free extracts involves the same MAP kinase kinase as is activated by progesterone treatment.

Activation of MAP kinase and the activation of MPF are separable events in cell-free extracts

Analysis of immunoblots of *malE–mos*-treated oocyte extracts with a monoclonal antibody against *Xenopus* p34^{cdc2} showed two bands, whose relative proportions did not change during the incubation with *malE–mos* (Figure 2C). The upper band presumably represents the tyrosine-phosphorylated, cyclin B-bound p34^{cdc2} component of pre-MPF (Dunphy and Newport, 1989; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991), although the anti-phosphotyrosine antibodies we have tested do not recognize this band with the same sensitivity as they recognize the activated form of MAP kinase. This may be due to the presence of an adjacent phosphothreonine residue.

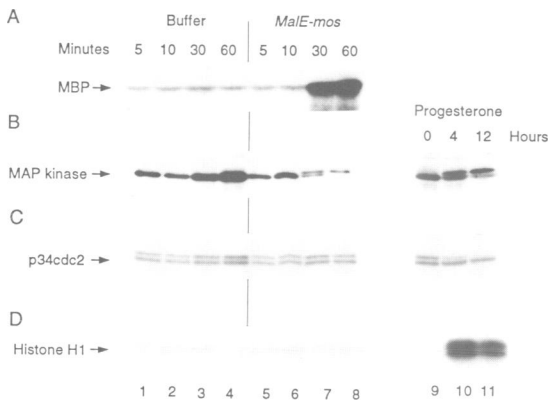

Fig. 2. Effect of *malE–mos* protein on endogenous MAP kinase and p34^{cdc2}. Cell-free extracts were incubated with buffer (lanes 1–4) or 25 μg/ml of *malE–mos* (lanes 5–8) for the indicated times. As a control, oocytes unstimulated (lane 9) or maturated with progesterone for 4 h (lane 10) or 12 h (lane 11) were used. **A.** MBP kinase assay. **B.** Immunoblot with anti-MAP kinase antibody, showing the mobility shift that accompanies activation. **C.** An immunoblot with anti-p34^{cdc2} monoclonal antibody 3E1 of the same blot as shown in panel B. **D.** Histone H1 kinase assay.

Progesterone-matured oocytes and eggs displayed a single, lower p34^{cdc2} band (Figure 2C, lanes 10 and 11) corresponding to the active form of MPF. Failure to dephosphorylate p34^{cdc2} in response to added *malE–mos* was consistent with the absence of histone H1 kinase activity in the *malE–mos*-incubated extracts (Figures 1A and 2D).

These experiments show that *malE–mos* is able to trigger MAP kinase activation in cell-free extracts, presumably by initiating a protein kinase cascade. This process can occur without the activation of MPF, even though p34^{cdc2} is present at high levels. To check that it was in principle possible to activate cyclin B–p34^{cdc2} in our extracts, we added either recombinant cyclin A protein or okadaic acid, both of which have previously been shown to activate MPF in intact *Xenopus* oocytes (Goris *et al.*, 1989; Roy *et al.*, 1991). Both of these agents caused large increases in histone H1 kinase activity and MBP kinase activity (Figure 4A, lanes 5–10), and both led to the disappearance of the upper band of p34^{cdc2} and the complete upward mobility shift in MAP kinase (Figure 4B). There was clearly no dominant inhibitor of MPF activation present in our extracts. The high level of MBP phosphorylation in okadaic acid-treated extracts is partly due to the activation of cyclin B–p34^{cdc2} kinase, which can itself phosphorylate MBP (Shibuya *et al.*, 1992a). It is also possible that other MBP kinases, yet to be identified in *Xenopus* oocytes, are activated by okadaic acid.

The above results demonstrated that the activation of MAP kinase by *malE–mos* in cell-free extracts could occur without being rapidly followed by the activation of MPF. We next asked whether activation of cyclin B–p34^{cdc2} kinase could be promoted by the activated MAP kinase after longer incubation times. When oocyte extracts were incubated with *malE–mos*, MAP kinase was reproducibly activated by the recombinant c-*mos* protein in ~30–60 min, and the MAP kinase activity remained high for at least 6 h (Figure 5). The activation of cyclin B–p34^{cdc2} kinase was variable. In some extracts (Figure 5A and B), histone H1 kinase activity appeared ~2–3 h after *malE–mos* addition,

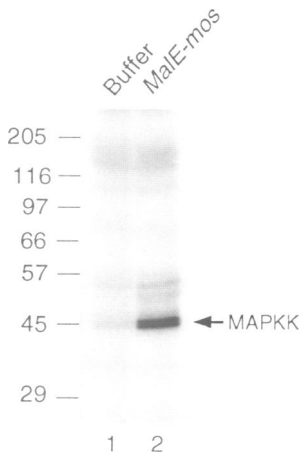

Fig. 3. Activation of MAP kinase kinase by *malE–mos* in oocyte extracts. Cell-free extracts were incubated with buffer (lane 1) or 32 μg/ml of *malE–mos* (lane 2) for 45 min. After 20-fold dilution in H1 kinase buffer they were immunoprecipitated with anti-MAP kinase kinase antiserum provided by Drs E.Nishida and Y.Gotoh. Immunoprecipitates were incubated with [γ-^{32}P]ATP as described by Kosako *et al.* (1992), except that the buffer also contained 10 mM $MgCl_2$. The samples were analysed by SDS–PAGE (15% acrylamide) and autoradiography.

and concomitantly, the upper band of p34^{cdc2} was lost, indicating MPF activation. Occasionally, control aliquots from these extracts also showed spontaneous activation of cyclin B−p34^{cdc2} kinase (Figure 5B, lane 3), but when *malE−mos* was added, it accelerated this activation of MPF (Figure 5B, lane 7). No detectable spontaneous activation of MAP kinase occurred in the extracts in which MPF was spontaneously activated. In the second kind of extract (Figure 5C and D), activation of MPF did follow the activation of MAP kinase produced by added *malE−mos*, but control extracts showed no spontaneous activation of either kinase. Finally, Figure 5E and F shows that in some extracts, the rapid activation of MAP kinase by *malE−mos* was never followed by activation of cyclin B−p34^{cdc2} kinase, even when incubations were continued for up to 6 h. In all three kinds of extracts, however, addition of okadaic acid or recombinant cyclin A reproducibly activated both the endogenous cyclin B−p34^{cdc2} kinase and MAP kinase (see Figure 4, lanes 7−10). The failure of active MAP kinase to activate MPF in cell-free extracts has also been reported by Shibuya *et al.* (1992b).

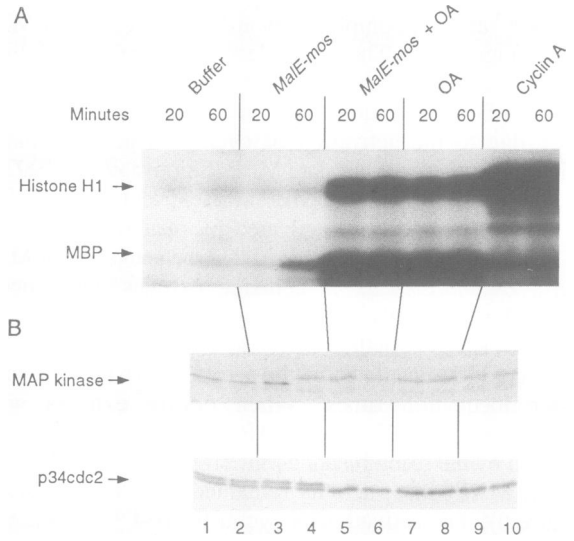

Fig. 4. Cyclin A and okadaic acid activate endogenous p34^{cdc2} and MAP kinases in oocyte extracts. Cell-free extracts were incubated with: buffer, lanes 1 and 2; *malE−mos* (25 μg/ml), lanes 3 and 4; *malE−mos* plus okadaic acid, lanes 5 and 6; okadaic acid (OA, 10 μM), lanes 7 and 8; cyclin A (55 μg/ml, 0.78 μM), lanes 9 and 10. **A.** Kinase activity against exogenous histone H1 and MBP. **B.** Immunoblots of the same samples with anti-MAP kinase and anti-p34^{cdc2} antibodies as indicated.

Addition of malE−mos to interphase extracts activates MAP kinase

Fertilization of *Xenopus* eggs is followed by cyclin destruction and inactivation of MPF, and loss of MAP kinase activity occurs ~30 min later (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991b). Figure 6 shows that a similar delayed loss of MAP kinase following the loss of histone H1 kinase activity of MPF occurs in cell-free extracts during the transition from metaphase to interphase induced by Ca^{2+} in CSF-arrested egg extracts. Considering that c-*mos* is destroyed at about the same time as MAP kinase activity declines (Lorca *et al.*, 1991; Watanabe *et al.*, 1991), we next asked whether addition of *malE−mos* to interphase extracts in which MAP kinase was inactive and the endogenous c-*mos* has been lost, could re-activate MAP kinase. As shown in Figure 7, *malE−mos* induced activation of the endogenous MAP kinase in interphase extracts, as indicated both by increased MBP kinase activity and by the typical change in electrophoretic mobility of the MAP kinase protein. Some activation could be seen as early as 15 min and conversion of the 42 kDa band to the upper form was complete by 45 min. No H1 kinase activity was detected in these conditions. As we found in oocyte extracts, addition of recombinant cyclin A reproducibly activated both H1 kinase activity and MAP kinase in these interphase extracts (data not shown). Therefore, the *in vitro* activation of MAP kinase by *malE−mos* is not a peculiarity of oocyte extracts. Moreover, using anti-*malE* antibodies, we could recover *malE−mos* with autophosphorylation activity after a 15−30 min incubation in the interphase extracts (data not shown). Thus, the recombinant *malE−mos* protein can be activated in cell-free extracts from both oocytes and activated eggs and leads to activation of MAP kinase in both cases.

MalE−mos can maintain the activity of MAP kinase under conditions where it would otherwise show transient activation

To strengthen further the functional relationship between c-*mos* and MAP kinase, we asked whether *malE−mos* could

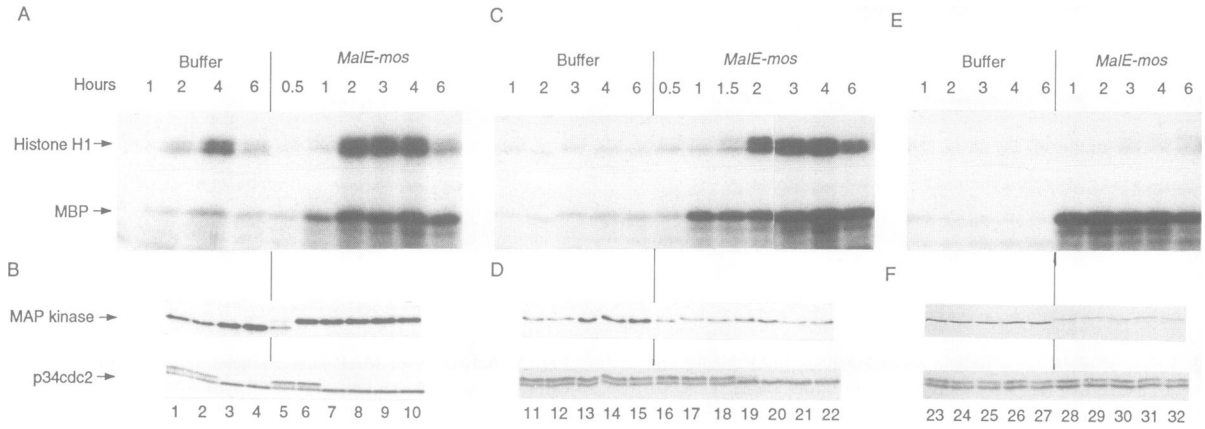

Fig. 5 Activation of histone H1 kinase and mobility shifts of p34^{cdc2} and MAP kinase in cell-free extracts of *Xenopus* oocytes. Cell-free extracts prepared from three different batches of oocytes were incubated with buffer (lanes 1−4, 11−15 and 23−27) or 32 μg/ml of *malE−mos* (lanes 5−10, 16−22 and 28−32) for the times indicated at the top of the Figure. **A, C** and **E.** Kinase activity against exogenous histone H1 and MBP. **B, D** and **F.** Immunoblots of the same samples with anti-MAP kinase and anti-p34^{cdc2} antibodies.

maintain MAP kinase activity under conditions where it was normally transient. For this purpose, we prepared extracts from oocytes that had been exposed to progesterone for 30 min. This short exposure to progesterone was sufficient to activate high levels of histone H1 and MAP kinase activity in the extracts, but by 6 h of incubation at 23°C, both activities had declined to low levels (Figure 8, lanes 1–4). The addition of *malE–mos* to these extracts slightly delayed the inactivation of histone H1 kinase and maintained high activity of MAP kinase for at least 6 h (Figure 8, lanes 5–8). Very similar results were observed when 1 vol of CSF-arrested egg extract was added to 6 vol of oocyte extract. This produced rapid (but transient) activation of both MPF and MAP kinase activities. The activity of MPF was lost at ~1.5–2 h after mixing, and MAP kinase turned off after 3–4 h. Again, however, the activity of MAP kinase (but not of MPF) was maintained at a high level by the addition of *malE–mos* (data not shown). Thus, *malE–mos* is able to activate MAP kinase in a variety of cell-free extracts and can also maintain MAP kinase activity under conditions where it would normally turn off.

In intact oocytes, MAP kinase and MPF turn on together

These results show that the c-*mos* protein kinase can activate MAP kinase in cell-free extracts. But what happens when intact oocytes are stimulated with progesterone? Since c-*mos* starts to be synthesized soon after hormonal stimulation, MAP kinase activation could in principle precede cyclin B–p34^{cdc2} kinase activation. Several laboratories have already examined this point in oocytes, taking pooled samples every hour after stimulation with progesterone (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991b; Posada *et al.*, 1991). All these authors report that MAP kinase was activated at about the same time as cyclin B–p34^{cdc2} kinase. To establish a more detailed correlation in intact oocytes between MAP kinase activation and cyclin B–p34^{cdc2} kinase activation, we incubated oocytes with progesterone and sampled single oocytes every 10 min. We observed a precise correlation between MAP kinase activation and cyclin B–p34^{cdc2} activation in every oocyte analysed (Figure 9). Either both kinases were activated or neither, and in the oocyte sampled at 4 h, both kinases showed signs of partial activation. In no case was one and not the other turned on. Therefore, although we can separate the activation of MAP kinase and the activation of MPF in cell-free extracts, during progesterone induction of maturation, they appear to be tightly coupled.

Activation of MAP kinase without MPF does not cause nuclear envelope breakdown or chromosome condensation

Since we were able to activate MAP kinase in interphase egg extracts, and it is known that MAP kinase can phosphorylate at least some of the same substrates as MPF, we asked whether activation of MAP kinase would suffice to cause any of the changes seen when cells enter mitosis. We added nuclei to extracts prepared as described by Blow and Laskey (1986). No significant difference was observed between control and *malE–mos*-treated extracts over a 2 h incubation period at 23°C, in spite of the activation of MAP kinase by *malE–mos* with the same kinetics as in Figure 7. We conclude that MAP kinase is unable to substitute for MPF. To make sure that these extracts could undergo nuclear envelope breakdown and chromosome

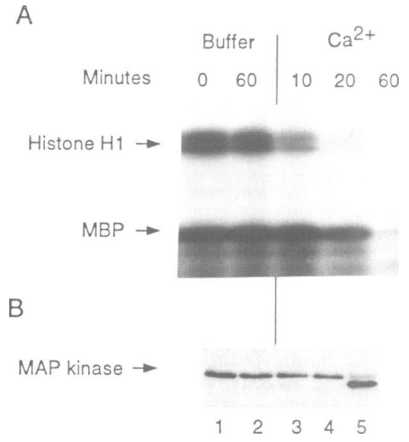

Fig. 6. MAP kinase activity declines after the transition from metaphase to interphase. CSF-arrested eggs extracts were incubated with 0.4 mM $CaCl_2$ for the indicated times. **A.** Kinase activity against exogenous histone H1 and MBP. **B.** Immunoblot of the same samples with anti-MAP kinase antibodies.

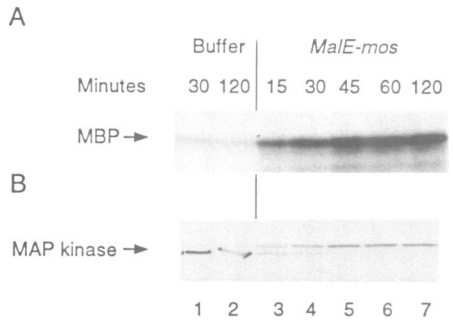

Fig. 7. Activation of MAP kinase by *malE–mos* in interphase extracts. Interphase extracts were incubated with buffer (lanes 1 and 2) or 40 μg/ml of *malE–mos* (lanes 3–7) for the indicated times. **A.** MBP kinase assay. **B.** Immunoblot with anti-MAP kinase antibodies.

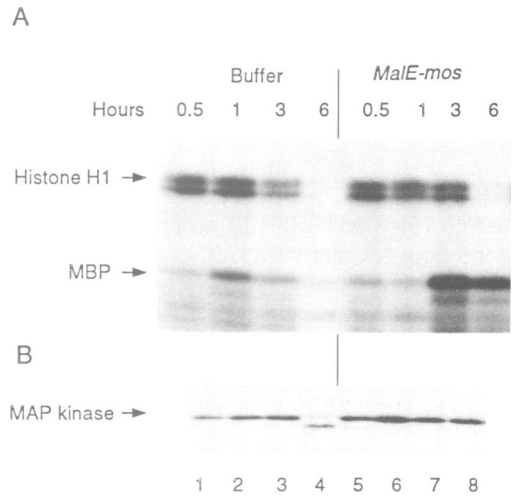

Fig. 8. *MalE–mos* prevents loss of MAP kinase activity in oocyte extracts. Cell-free extracts prepared from oocytes treated with progesterone for 30 min were incubated with buffer or 25 μg/ml of *malE–mos* for the indicated times. **A.** Kinase activity against exogenous histone H1 and MBP. **B.** Immunoblot of the same samples with anti-MAP kinase antibodies.

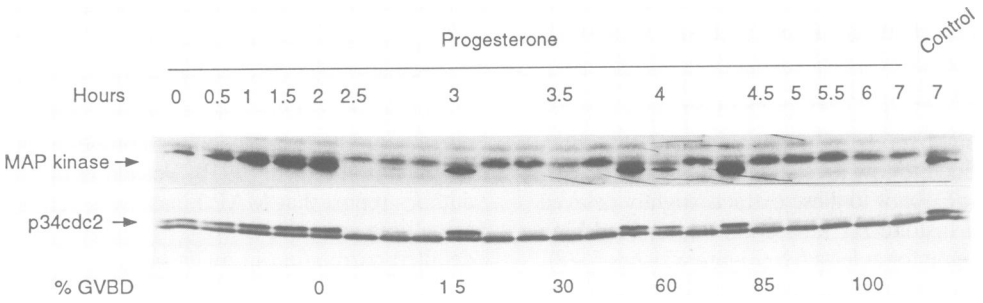

Fig. 9. Activation of MAP kinase and p34^{cdc2} during progesterone stimulation of *Xenopus* oocytes. At the indicated times after progesterone addition (samples being taken at 10 min intervals from 2.5–4.5 h), single oocytes were frozen on dry ice. These samples were analysed by SDS–PAGE and sequential immunoblotting on the same Immobilon sheet, first with anti-MAP kinase and second with anti-p34^{cdc2} antibodies as described in Materials and methods. The percentage of maturation (GVBD) was analysed in the group of oocytes by scoring the presence of a white spot in the animal pole of the oocytes in the population as a whole.

condensation, either cyclin A or cyclin B were added. Either of these proteins caused increased histone H1 kinase activity, nuclear envelope breakdown and chromosome condensation (Murray and Kirschner, 1989; Roy *et al.*, 1991).

Discussion

In this paper we show that bacterially produced c-*mos* protein kinase rapidly activates MAP kinase in cell-free extracts of *Xenopus* oocytes and eggs. This activation probably occurs via the 45 kDa MAP kinase kinase described by the group of Nishida and Gotoh (Kosako *et al.*, 1992; Matsuda *et al.*, 1992), since we show that this enzyme is also activated by *malE–mos*. While it is likely that c-*mos* directly phosphorylates MAP kinase kinase, as has been proposed in the case of c-*raf* (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992), our data do not exclude the possibility that c-*mos* acts further up the MAP kinase activation pathway. We also show that the activation of MAP kinase by c-*mos* can occur independently of the activation of MPF, even though purified MPF can activate MAP kinase kinase and MAP kinase (Gotoh *et al.*, 1991a; Matsuda *et al.*, 1992). Oncogenic *ras* proteins are also capable of activating both MAP kinase kinase and MAP kinase in *Xenopus* oocytes. However, the *ras*-activated pathway does not appear to require c-*mos*, because it can occur in cycloheximide-treated oocytes and in cell-free extracts, and is also independent of MPF activation (Hattori *et al.*, 1992; Pomerance *et al.*, 1992; Shibuya *et al.*, 1992b; Nebreda *et al.*, 1993). It thus appears that MAP kinase can be activated in *Xenopus* oocytes by several pathways, whose interconnections are not yet clear.

MAP kinase normally loses activity 30 min after fertilization of *Xenopus* eggs (Ferrell *et al.*, 1991), which coincides with the time that c-*mos* is normally lost by proteolysis in these cells (Lorca *et al.*, 1991; Watanabe *et al.*, 1991). As we show in this paper, loss of MAP kinase activity from *Xenopus* extracts can be prevented by addition of *malE–mos* protein, both in transiently activated oocyte extracts and in extracts of activated eggs. This correlation between the presence of c-*mos* and the activity of MAP kinase suggests that the maintenance of high MAP kinase activity during meiosis may be an important role of c-*mos* in *Xenopus* oocytes.

In view of the well known association between cytostatic factor (CSF) and c-*mos* (Sagata *et al.*, 1989b), we were somewhat surprised that *malE–mos* was never able to maintain MPF activity in any of our experiments. Only in some experiments was a slight delay in the loss of histone H1 kinase activity in *malE–mos*-treated samples observed, compared with controls without it. The bacterially expressed *malE–mos* was clearly active, because it was capable of turning on and maintaining the activity of MAP kinase. We therefore suppose that an additional factor to c-*mos*, which would normally be present in unfertilized eggs, must be missing from the oocyte extracts. Another possibility, however, is that the inactivation of MPF between meiosis I and meiosis II is insensitive to the action of CSF, since our assays only test this particular cell cycle transition. It is obviously of interest to know whether or not the cytostatic activity of c-*mos* in meiotic metaphase II depends on its ability to activate MAP kinase. Indeed, it may be significant that oncogenic *ras*, which can activate MAP kinase in interphase extracts (Shibuya *et al.*, 1992b), has been reported to arrest embryonic cell cleavage in mitotic metaphase (Daar *et al.*, 1991).

Microinjection of c-*mos* protein and mRNA can induce MPF activation in intact *Xenopus* oocytes (Freeman *et al.*, 1989; Sagata *et al.*, 1989a; Yew *et al.*, 1992). In many preparations of oocyte extracts we indeed observed the activation of cyclin B–p34^{cdc2} kinase (MPF) after addition of *malE–mos*, although this always required significantly longer incubations than the activation of MAP kinase. In some extracts, however, activation of MAP kinase by *malE–mos* was not followed by the activation of cyclin B–p34^{cdc2} kinase at any time during the course of a 6 h incubation. These differences may be due to variations in the quality of different batches of oocytes. The above observation that in cell-free extracts activation of cyclin B–p34^{cdc2} kinase by *malE–mos* always occurs later than MAP kinase activation (or not at all), is surprising considering that (i) progesterone treatment of oocytes activates MAP kinase and cyclin B–p34^{cdc2} kinase within 10 min of one another (Figure 9) and (ii) synthesis of c-*mos* is required for progesterone-induced maturation (Sagata *et al.*, 1988). It thus appears that in cell-free extracts cyclin B–p34^{cdc2} kinase is usually more tightly 'locked' in the inactive state than in intact oocytes. This is in agreement with the observation in similar extracts that oncogenic *ras* protein can activate MAP kinase, but not cyclin B–p34^{cdc2} kinase (Shibuya *et al.*, 1992b). This may be due to loss of crucial components necessary for the activation of MPF

during the preparation of the extracts. An alternative possibility is suggested by the work of Yew *et al.* (1992), who found that when protein synthesis was inhibited, low doses of *malE−mos* could only induce oocyte maturation if the oocytes were also exposed to progesterone. These results suggest that physiological activation of MPF during oocyte maturation requires another signal initiated by progesterone in addition to stimulation of the synthesis of c-*mos*. The pathway(s) required for progesterone-induced activation of MPF in *Xenopus* oocytes have yet to be identified. We cannot tell whether the requirement of c-*mos* for MPF activation (Sagata *et al.*, 1988; Pickham *et al.*, 1992), is due to the activation of MAP kinase itself by c-*mos* or some other property, such as its ability to phosphorylate tubulin (Zhou *et al.*, 1991).

The synchronous activation of MAP kinase and cyclin B−p34^{cdc2} kinase during progesterone induction of maturation (we never found examples of one kinase being activated without the other), suggests to us that the natural activation of these protein kinases may depend on a common regulatory event. Considering the ability of okadaic acid to activate MPF (Goris *et al.*, 1989), and the ability of phosphatase 2A to both inhibit MPF activation (Lee *et al.*, 1991) and inactivate MAP kinase kinase and MAP kinase (Anderson *et al.*, 1990; Gomez and Cohen, 1991; Matsuda *et al.*, 1992), there may be an important role for protein phosphatases in this regulation.

Finally, we would point out that if c-*mos* can activate the MAP kinase pathway in somatic cells, it would suggest a new explanation for c-*mos* being a transforming oncogene. The c-*mos* proto-oncogene is normally only expressed at significant levels in germ cells (Propst *et al.*, 1987; Sagata *et al.*, 1988). Provided that c-*mos* can activate MAP kinase in somatic cells (an important point we have not tested), it ought to provide essentially the same inappropriate signal as do oncogenic *ras* and *raf*, both of which clearly activate MAP kinase (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Leevers and Marshall, 1992). Under normal conditions, MAP kinase is transiently activated by a variety of physiological signals (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Kosako *et al.*, 1992; Nakielny *et al.*, 1992). It appears that in turn, MAP kinase can elevate the level and activity of transcription factors such as *fos* and *jun* (Pulverer *et al.*, 1991; Gille *et al.*, 1992). MAP kinase is now recognized as an important element in several signal transduction pathways in eukaryotes from yeast to man (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Sprague, 1992; Stevenson *et al.*, 1992). It is easy to understand why the inappropriate overexpression of MAP kinase activity could cause cells to ignore regulatory signals from their environment.

Materials and methods

Preparation of malE−mos protein

For the preparation of a *malE−mos* recombinant protein, the *Xenopus* c-*mos* proto-oncogene (Sagata *et al.*, 1988) was fused in-frame to the maltose-binding protein of *E.coli*. We generated a 1.2 kb DNA fragment by PCR, which comprised the complete *Xenopus* c-*mos* coding region with an *Eco*RI site before the first ATG (sequence: GAA TTC ACC ATG) and a *Hin*dIII site 154 base pairs downstream of the stop codon. The 5′ oligonucleotide used was 5′-GATCCCCGGGCGAATTCACCATGGCTTC-3′; the 3′ oligonucleotide was 5′-GACAAGGAGTGAAGCTTCCTCACTAGTGC-3′. After digestion with *Eco*RI and *Hin*dIII, the PCR product was inserted between the *Eco*RI and *Hin*dIII sites of pMAL-cRI (New England Biolabs), and the resulting construct was used to transform *E. coli* TG-1 cells. The *malE−mos* fusion protein was induced by IPTG and purified from the soluble fraction as previously described by Yew *et al.* (1992). Final *malE−mos* preparations were dialysed against 20 mM Tris, pH 7.5 and 50 mM NaCl, and stored at −70°C.

Preparation of cell-free extracts

Concentrated cell-free extracts were prepared from *Xenopus* oocytes essentially as described by Murray (1991) for 'CSF-arrested' extracts of eggs. The dissected ovaries were treated with 1 mg/ml of collagenase A (Boehringer Mannheim) in 5 mM HEPES, 96 mM NaCl, 2 mM KCl and 1 mM $MgCl_2$ (pH 7.8) for 2−4 h at room temperature and then extensively washed with modified Barth's medium [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, (pH 7.6)]. Stage V and VI oocytes were sorted by hand and washed 20−30 times in XB buffer (Murray, 1991) before preparation of the extracts. Aliquots were frozen in liquid nitrogen and stored at −70°C. Typical protein concentrations in these extracts were 40−60 mg/ml, as determined by the Bradford assay (Bio-Rad). We found that more dilute extracts, obtained by homogenizing oocytes in 2 vol of XB buffer, were unresponsive to *malE−mos*. For the preparation of interphase extracts, CSF-arrested eggs extracts (Murray, 1991) were incubated with 100 μg/ml cycloheximide for 10 min, followed by 0.4 mM $CaCl_2$ for 60 min and 0.4 mM EGTA for 5 min at 23°C.

Oocyte maturation

Oocytes were incubated with 5 μg/ml of progesterone (Sigma) in modified Barth's medium for the indicated times, and then frozen on dry ice. For the preparation of lysates, oocytes were homogenized in 20 μl per oocyte of ice-cold H1 kinase buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$) supplemented with 0.5 mM Na_3VO_4 and protease inhibitors (Murray, 1991). The lysates were centrifuged at 10 000 *g* for 8 min, and 8 μl of the cleared supernatant were used for H1 kinase or immunoblot analysis.

Kinase assays

For kinase activation assays, extracts were incubated with *malE−mos* at 23°C. Usually, 1 vol of *malE−mos* preparation was mixed with 4−8 vol of concentrated extracts. Samples were diluted 1:12 in ice-cold H1 kinase buffer (see above) and frozen on dry ice. Histone H1 kinase activity was assayed in a final volume of 12 μl of H1 kinase buffer, containing 0.3−1 μl of concentrated extract or 8 μl of oocyte lysate, 2 μg of histone H1 (Boehringer Mannheim) and 100 μM [γ-^{32}P]ATP (5000 c.p.m./pmol), for 15 min at room temperature. Phosphorylated proteins were analysed by SDS−PAGE (15% acrylamide) and autoradiography. For MBP kinase, 5 μl of 12-fold diluted extracts were assayed in a final volume of 40 μl with 40 μg of MBP (Sigma) and 50 μM [γ-^{32}P]ATP (5000 c.p.m./pmol) as described by Erickson *et al.* (1990). Aliquots of 30 μl were spotted on P81 phosphocellulose discs, which were washed and counted in a scintillation counter by Cerenkov radiation. In some cases, an aliquot of the reaction (5 μl) was analysed by SDS−PAGE (20% acrylamide) and autoradiography. When kinase activities on histone H1 and MBP were assayed together, 12 μl of H1 kinase buffer containing 4 μl of 1:12 diluted extract were incubated with 1 μg of histone H1, 3 μg of MBP, 50 μM [γ-^{32}P]ATP (5500 c.p.m./pmol) and 5 μM protein kinase A inhibitor peptide (Sigma) for 15 min at room temperature. Phosphorylation was analysed by SDS−PAGE (20% acrylamide) and autoradiography.

Immunoblotting

After SDS−PAGE (15% acrylamide) using 0.2−0.4 μl of concentrated extracts or 8 μl of oocyte lysates per lane, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). MAP kinase was detected using a monoclonal antibody (Zymed) that recognizes both ERK-1 and ERK-2 MAP kinases from a variety of sources. This antibody recognized recombinant *Xenopus* MAP kinase (not shown). p34^{cdc2} was detected using the 3E1 monoclonal antibody, which was raised against the C-terminal 219 residues of bacterially expressed *Xenopus* p34^{cdc2} protein (J.Gannon, R.Poon and T.Hunt, unpublished). The immunoblots were probed sequentially with both antibodies, first with the anti-MAP kinase antibody using ECL detection (Amersham International) followed by a further round of blocking and incubation with anti-p34^{cdc2} antibody, whose binding was detected with alkaline phosphatase staining.

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References

Ahn,N.G., Seger,R., Bratlien,R.L., Ditlz,C.D., Tonks,N.K. and Krebs,E.G. (1991) *J. Biol. Chem.*, **266**, 4220–4227.
Alessandrini,A., Crews,C.M. and Erickson,R.L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8200–8204.
Anderson,N.G., Maller,J.L., Tonks,N.K. and Sturgill,T.W. (1990) *Nature*, **343**, 651–653.
Barrett,C.B., Erikson,E. and Maller,J.L. (1992) *J. Biol. Chem.*, **267**, 4408–4415.
Birchmeier,C., Broek,D. and Wigler,M. (1985) *Cell*, **43**, 615–621.
Blow,J.J. and Laskey,R.A. (1986) *Cell*, **47**, 577–587.
Cairns,B.R., Ramer,S.W. and Kornberg,R.D. (1992) *Genes Dev.*, **6**, 1305–1318.
Cicirelli,M.F., Pelech,S.L. and Krebs,E.G. (1988) *J. Biol. Chem.*, **263**, 2009–2019.
Cyert,M.S. and Kirschner,M.W. (1988) *Cell*, **53**, 185–195.
Daar,I., Nebreda,A.R., Yew,N., Sass,P., Paules,R., Santos,E., Wigler,M. and Vande Woude,G.F. (1991) *Science*, **253**, 74–76.
Dent,P., Haser,W., Haystead,T.A.J., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) *Science*, **257**, 1404–1407.
Dunphy,W. and Newport,J. (1989) *Cell*, **58**, 181–191.
Erickson,A.K., Payne,D.M., Martino,P.A., Rossomando,A.J., Shabanowitz,J., Weber,M.J., Hunt,D.F. and Sturgill,T.W. (1990) *J. Biol. Chem.*, **265**, 19728–19735.
Ferrell,J.E., Wu,M., Gerhart,J.C. and Martin,G.S. (1991) *Mol. Cell. Biol.*, **11**, 1965–1971.
Freeman,R.S., Pickham,K.M., Kanki,J.P., Lee,B.A., Pena,S.V. and Donoghue,D.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5805–5809.
Freeman,R.S., Kanki,J.P., Ballantyne,S.M., Pickham,K.M. and Donoghue,D.J. (1990) *J. Cell Biol.*, **111**, 533–541.
Gartner,A., Nasmyth,K. and Ammerer,G. (1992) *Genes Dev.*, **6**, 1280–1292.
Gautier,J. and Maller,J.L. (1991) *EMBO J.*, **10**, 177–182.
Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197–211.
Gille,H., Sharrocks,A.D. and Shaw,P.E. (1992) *Nature*, **358**, 414–417.
Gomez,N. and Cohen,P. (1991) *Nature*, **353**, 170–173.
Goris,J., Hermann,J., Hendrix,P., Ozon,R. and Merlevede,W. (1989) *FEBS Lett.*, **245**, 91–94.
Gotoh,Y. *et al.* (1991a) *EMBO J.*, **10**, 2661–2668.
Gotoh,Y., Nishida,E., Matsuda,S., Shiina,N., Kosako,H., Shiokawa,K., Akiyama,T., Ohta,K. and Sakai,H. (1991b) *Nature*, **349**, 251–254.
Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39–45.
Hattori,S., Fukuda,M., Yamashita,T., Nakamura,S., Gotoh,Y. and Nishida,E. (1992) *J. Biol. Chem.*, **267**, 20346–20351.
Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) *Cell*, **71**, 335–342.
Izumi,T., Walker,D.H. and Maller,J.L. (1992) *Mol. Biol. Cell*, **3**, 927–939.
Kirschner,M., Newport,J. and Gerhart,J. (1985) *Trends Genet.*, **1**, 41–47.
Kobayashi,H., Golsteyn,R., Poon,R., Stewart,E., Gannon,J., Minshull,J., Smith,R. and Hunt,T. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 437–447.
Kosako,H., Gotoh,Y., Matsuda,S., Ishikawa,M. and Nishida,E. (1992) *EMBO J.*, **11**, 2903–2908.
Kumagai,A. and Dunphy,W.G. (1991) *Cell*, **64**, 903–914.
Kumagai,A. and Dunphy,W.G. (1992) *Cell*, **70**, 139–151.
Kyriakis,J.M., App,H., Zhang,Z.-F., Banerjee,P., Brautigan,D.L., Rapp,U. and Avruch,J. (1992) *Nature*, **358**, 417–421.
Lane,H.A., Morley,S.J., Dorée,M., Kozma,S.C. and Thomas,G. (1992) *EMBO J.*, **11**, 1743–1749.
Lee,T.H., Solomon,M.J., Mumby,M.C. and Kirschner,M.W. (1991) *Cell*, **64**, 415–423.
Leevers,S.J. and Marshall,C.J. (1992) *Trends Cell Biol.*, **2**, 283–286.
Lohka,M. and Masui,Y. (1984) *J. Cell Biol.*, **98**, 1222–1230.
Lorca,T., Galas,S., Fesquet,D., Devault,A., Cavadore,J.-C. and Dorée,M. (1991) *EMBO J.*, **10**, 2087–2093.
Maller,J.L. (1990) *Biochemistry*, **29**, 3157–3166.
Matsuda,S., Kosako,H., Takenaka,K., Moriyama,K., Sakai,H., Akiyama,T., Gotoh,Y. and Nishida,E. (1992) *EMBO J.*, **11**, 973–982.
Millar,J.B. and Russell,P. (1992) *Cell*, **68**, 407–410.
Minshull,J., Murray,A., Colman,A. and Hunt,T. (1991) *J. Cell Biol.*, **114**, 767–772.
Murray,A.W. (1991) *Methods Cell Biol.*, **36**, 573–597.
Murray,A.W. and Kirschner,M.W. (1989) *Nature*, **339**, 275–280.
Nakielny,S., Cohen,P., Wu,J. and Sturgill,T. (1992) *EMBO J.*, **11**, 2123–2129.
Nebreda,A.R., Porras,A. and Santos,E. (1993) *Oncogene*, **8**, 467–477.
Paris,J., Le Guellec,R., Couturier,A., Le Guellec,K., Omilli,F., Camonis,J., MacNeill,S. and Philippe,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1039–1043.
Pickham,K.M., Meyer,A.N., Li,J. and Donoghue,D.J. (1992) *Mol. Cell. Biol.*, **12**, 3192–3203.
Pomerance,M., Schweighoffer,F., Tocque,B. and Pierre,M. (1992) *J. Biol. Chem.*, **267**, 16155–16160.
Posada,J. and Cooper,J.A. (1992) *Science*, **255**, 212–215.
Posada,J., Sanghera,J., Pelech,S., Aebersold,R. and Cooper,J.A. (1991) *Mol. Cell. Biol.*, **11**, 2517–2528.
Propst,F., Rosenberg,M.P., Iyer,A., Kaul,K. and Vande Woude,G.F. (1987) *Mol. Cell. Biol.*, **7**, 1629–1637.
Pulverer,B.J., Kyriakis,J.M., Avruch,J., Nikolakaki,E. and Woodgett,J.R. (1991) *Nature*, **353**, 670–674.
Roy,L.M., Singh,B., Gautier,J., Arlinghaus,R.B., Nordeen,S.K. and Maller,J.L. (1990) *Cell*, **61**, 825–831.
Roy,L.M., Swenson,K.I., Walker,D.H., Gabrielli,B.G., Li,R.S., Piwnica,W.H. and Maller,J.L. (1991) *J. Cell Biol.*, **113**, 507–514.
Sagata,N., Oskarsson,M., Copeland,T., Brumbaugh,J. and Vande Woude,G.F. (1988) *Nature*, **335**, 519–526.
Sagata,N., Daar,I., Oskarsson,M., Showalter,S.D. and Vande Woude,G.F. (1989a) *Science*, **245**, 643–646.
Sagata,N., Watanabe,N., Vande Woude,G.F. and Ikawa,Y. (1989b) *Nature*, **342**, 512–518.
Shibuya,E.K., Boulton,T.G., Cobb,M.C. and Ruderman,J.V. (1992a) *EMBO J.*, **11**, 3963–3975.
Shibuya,E.K., Polverino,A.J., Chang,E., Wigler,M. and Ruderman,J.V. (1992b) *Proc. Natl. Acad. Sci. USA*, **89**, 9831–9835.
Sprague,G.F.J. (1992) *Curr. Biol.*, **2**, 587–589.
Stevenson,B.J., Rhodes,N., Errede,B. and Sprague,G.F. (1992) *Genes Dev.*, **6**, 1293–1304.
Strausfeld,U., Labbé,J.C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Dorée,M. (1991) *Nature*, **351**, 242–245.
Sturgill,T.W. and Wu,J. (1991) *Biochim. Biophys. Acta*, **1092**, 350–357.
Watanabe,N., Hunt,T., Ikawa,Y. and Sagata,N. (1991) *Nature*, **352**, 247–248.
Xu,W., Ladner,K.J. and Smith,L.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 4573–4577.
Yew,N., Mellini,M.L. and Vande Woude,G.F. (1992) *Nature*, **355**, 649–652.
Zhou,R.P., Oskarsson,M., Paules,R.S., Schulz,N., Cleveland,D. and Vande Woude,G.F. (1991) *Science*, **251**, 671–675.

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