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Dependence of Mos-induced Cdc2 activation on MAP kinase function in a cell-free system

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The progression of G_2-arrested *Xenopus laevis* oocytes into meiotic M-phase is accompanied by the nearly simultaneous activation of p42 MAP kinase and Cdc2/cyclin B. This timing raises the possibility that the activation of one kinase might depend upon the other. Here we have examined whether Cdc2 activation requires p42 MAP kinase function. We have reconstituted Mos-induced Cdc2 activation in cell-free *Xenopus* oocyte extracts, and have found that Mos-induced Cdc2 activation requires active p42 MAP kinase, is inhibited by a MAP kinase phosphatase and is independent of protein synthesis. These findings indicate that p42 MAP kinase is an essential component of the M phase trigger in this system.

Keywords: Cdc2/MAP kinase/Mos/*Xenopus laevis*

Introduction

During oogenesis, *Xenopus laevis* oocytes grow to the size of ~200 000 somatic cells, and then arrest spontaneously in a G_2-like state with inactive Cdc2/cyclin B complexes. The arrested oocytes respond to the hormone progesterone by activating their stores of Cdc2/cyclin B and entering meiotic M phase. Studies of the G_2–M transition in oocytes have been important in identifying and establishing the relationships between M phase regulators, but there are still many gaps in our understanding of how progesterone brings about activation of Cdc2/cyclin B.

The transition into meiotic M phase requires synthesis of the Mos proto-oncoprotein (Sagata *et al.*, 1988, 1989; Yew *et al.*, 1992). Mos is present at very low levels in G_2 phase-arrested oocytes (Watanabe *et al.*, 1989). Progesterone causes the Mos mRNA to become polyadenylated (Sheets *et al.*, 1994, 1995), which results in an increase in the rate of Mos synthesis and the level of Mos protein (Watanabe *et al.*, 1989). Newly synthesized Mos becomes active, through a process that is poorly understood, and can then bring about the activation of Cdc2/cyclin B complexes (Yew *et al.*, 1992).

One plausible hypothesis for how Mos activates Cdc2/cyclin B is that it is through the intermediacy of the MAP kinase cascade. Mos can phosphorylate and activate MAP kinase kinase(s) (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993; Pham *et al.*, 1995; Resing *et al.*, 1995), which in turn can phosphorylate and activate MAP kinase. It is not known how many other targets Mos might have, but recent evidence suggests that MAP kinase kinase and MAP kinase are essential for Mos function in some systems (Kosako *et al.*, 1994a; Okazaki and Sagata, 1995).

Three previous studies have examined whether MAP kinase pathway components were important for Mos- or progesterone-induced Cdc2 activation, and have yielded conflicting results. One study used microinjected Mek antibodies to block Mek function, and found that the antibodies blocked progesterone-induced or Mos-induced p42 MAP kinase activation and Cdc2 activation (Kosako *et al.*, 1994b). This implies that Mek function is essential for Cdc2 activation, and, if MAP kinase is an essential mediator of Mek function, implicates MAP kinase as well. The other two studies used kinase-minus dominant-negative Raf mutants to examine whether Raf function was important for progesterone-induced germinal vesicle breakdown (GVBD) and Cdc2 activation. One found that dominant-negative Raf blocked progesterone-induced p42 MAP kinase and Cdc2 activation, consistent with the hypothesis that MAP kinase function was important for Cdc2 activation (Muslin *et al.*, 1993). The other found that dominant-negative Raf blocked progesterone-induced p42 MAP kinase activation, but only slightly delayed GVBD, implying that neither p42 MAP kinase nor (presumably) Mek function was essential for Cdc2 activation (Fabian *et al.*, 1993). It is difficult to reconcile these various conflicting findings.

Here we have used cell-free *Xenopus* oocyte extracts to examine the role of p42 MAP kinase in Mos-induced Cdc2/cyclin B activation. We demonstrate that Mos-induced Cdc2 activation can be reconstituted by supplementing extracts with modest amounts of p42 MAP kinase. Mos-induced Cdc2 activation requires p42 MAP kinase function, is inhibited by a MAP kinase phosphatase (Mkp-1) and does not require protein synthesis. These findings demonstrate that p42 MAP kinase plays an essential role in Cdc2 activation in this system.

Results and discussion

We set out to determine if p42 MAP kinase function is required for the activation of Cdc2 by Mos. Our initial approach was to attempt to interfere with p42 MAP kinase function in intact oocytes by various methods (antibody injection, expression or injection of kinase-minus MAP kinase mutants and antisense oligonucleotide injection). However, each of these methods failed to interfere substantially with p42 MAP kinase activation, as assessed by immunoblotting or kinase assays, or function, as assessed by phosphorylation of Rsk, a likely *in vivo* substrate of p42 MAP kinase (data not shown).

Reconstitution of Mos-dependent Cdc2 activation in extracts

We then turned to an oocyte extract system that faithfully recapitulates key aspects of the G_2–M transition *in vitro*

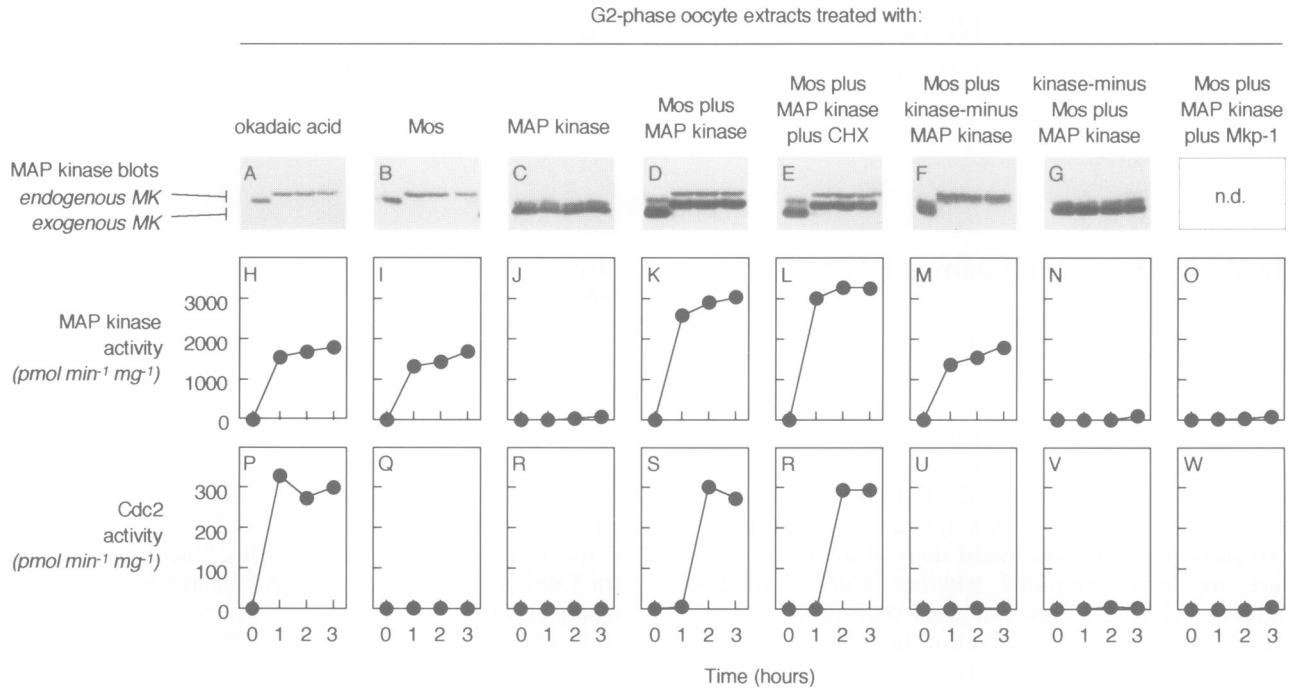

Fig. 1. MAP kinase function is required for Mos-induced Cdc2 activation in *Xenopus* oocyte extracts. Oocyte extracts were treated with one or more of the following at final concentrations as indicated: okadaic acid (5 μM), wild-type or kinase-minus (K90R) malE-Mos (Yew *et al.*, 1992) (2.8 ng/μl), wild-type or kinase-minus (K52R) rat Erk (Robbins *et al.*, 1993) (44 ng/μl), Mkp-1 (60 ng/μl) or cycloheximide (100 ng/μl). For the Mkp-1 incubations, the extracts were supplemented with dithiothreitol (2 mM) and bovine serum albumin (1 mg/ml). Samples were collected at various times and subjected to MAP kinase immunoblotting (top panels), MAP kinase assays (middle panels) and Cdc2 kinase assays (bottom panels). The data shown here are taken from two separate experiments using aliquots of one oocyte extract preparation. An MAP kinase blot was not performed for the Mkp-1-treated lysates whose kinase activities are shown in (**O**) and (**W**). The electrophoretically retarded bands represent phosphorylated p42 MAP kinase (Posada and Cooper, 1992). The results shown here are representative of four experiments.

(Shibuya *et al.*, 1992; Nebreda and Hunt, 1993). In agreement with previous reports, we found that Mos brought about activation of p42 MAP kinase but not of Cdc2 (Figure 1B, I and Q) (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993). One explanation for this finding could be that the link between Mos and Cdc2 is attenuated in extracts.

We tested this hypothesis by supplementing extracts with bacterially expressed p42 MAP kinase. We used rat Erk2/p42 MAP kinase because it can be distinguished from the endogenous *Xenopus* p42 MAP kinase by gel electrophoresis (Figure 1C–G). The rat protein is 95% identical in sequence to *Xenopus* p42 MAP kinase, and was found to be activated in Mos-treated extracts with the same kinetics as the endogenous p42 MAP kinase (Figure 1D–F).

As shown in Figure 1, addition of p42 MAP kinase restored the link between Mos and Cdc2; extracts to which p42 MAP kinase was added exhibited activation of Cdc2 in response to Mos. Modest amounts of added rat p42 MAP kinase (44 ng/μl, compared with endogenous levels of ~25 ng/μl) restored Mos-dependent Cdc2 activation in four out of nine extracts examined (Figure 1D, K and S). Because of the limited solubility of rat p42 MAP kinase and the need to avoid diluting the extracts, we were unable to determine whether higher concentrations of rat p42 MAP kinase would have restored Cdc2 activation in the other five extracts. The Mos-induced activation of Cdc2 typically lagged behind the activation of p42 MAP kinase (Figure 1K, L, S and R). Addition of p42 MAP kinase had no effect on the activity of endogenous p42 MAP kinase or Cdc2 in the absence of added Mos (Figure 1C, J and R), and had no effect when kinase-minus Mos was added (Figure 1G, N and V). The effect of the added p42 MAP kinase was distinctly non-linear; modest increases in p42 MAP kinase activity converted the extracts from exhibiting no Mos-induced Cdc2 activity to levels of Cdc2 activity similar to those seen in okadaic acid-treated extracts and progesterone-treated oocytes (150–300 pmol/min/mg; Figure 1P, S and R, and data not shown). A similar threshold effect is seen when Cdc2 is activated by addition of cyclin proteins in *Xenopus* interphase extracts; low levels of cyclin cause no activation, and then higher levels cause quantitative activation (Solomon *et al.*, 1990).

A kinase-minus mutant form of rat p42 MAP kinase was found to become phosphorylated in Mos-treated extracts, but not to restore Cdc2 activation (Figure 1F, M and U), demonstrating that the link between Mos and Cdc2 is dependent upon p42 MAP kinase activity. Addition of the dual specificity MAP kinase phosphatase Mkp-1 (Sun *et al.*, 1993) to the MAP kinase-supplemented extracts prevented Mos-induced MAP kinase activation and Cdc2 activation (Figure 1O and W), again arguing that the link between Mos and Cdc2 requires p42 MAP kinase activity.

New cyclin synthesis or activation of pre-existing Cdc2/cyclin B complexes?

It is thought that much of the increase in Cdc2 activity seen in progesterone-treated oocytes is due to dephos-

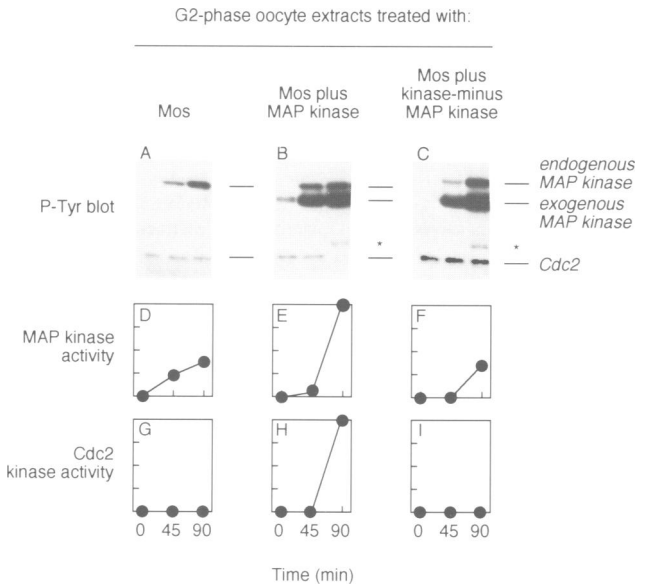

Fig. 2. Tyrosine phosphorylation of p42 MAP kinase and Cdc2 in samples treated with Mos and rat Erk2. Oocyte extracts were prepared and incubations carried out as described in the legend to Figure 1. The bands representing endogenous p42 MAP kinase, exogenous p42 MAP kinase and Cdc2 are denoted by bars. The band denoted by the asterisks represents truncated, bacterially expressed p42 MAP kinase. Kinase activities are expressed in arbitrary activity units.

phorylation of Cdc2's inhibitory Thr14 and Tyr15 phosphorylations in pre-existing Cdc2/cyclin complexes (Ferrell *et al.*, 1991; Jessus *et al.*, 1991). Some of the increase may also be due to new cyclin synthesis (Kobayashi *et al.*, 1991). We set out to determine whether either of these mechanisms were operating in the MAP kinase-supplemented extract system.

To determine if cyclin synthesis contributed to Mos-induced Cdc2 activation, we pre-treated extracts with cycloheximide at concentrations sufficient to inhibit protein synthesis maximally (Ferrell *et al.*, 1991). As shown in Figure 1E, L and R, cycloheximide treatment did not affect Cdc2 activation in the MAP kinase-supplemented extracts, indicating that neither cyclin nor any other protein needs to be synthesized. Similarly, cycloheximide treatment had no effect on okadaic acid-induced MAP kinase and Cdc2 activation, and no effect on Mos-induced MAP kinase activation (not shown).

To determine whether Mos and MAP kinase brought about dephosphorylation of the inhibitory sites on Cdc2, we assessed Cdc2 tyrosine phosphorylation by anti-phosphotyrosine immunoblotting. As shown in Figure 2B, the phosphotyrosine immunoreactivity of Cdc2 abruptly disappeared concomitantly with Cdc2 activation, consistent with the idea that Mos and MAP kinase bring about the disinhibition of inactive Cdc2/cyclin B. We also found that Mos and MAP kinase induced hyperphosphorylation of Cdc25 (not shown), which accompanies Cdc25 activation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). The hyperphosphorylation of Cdc25 coincided with Cdc2 activation. Whether the Mos- and MAP kinase-induced hyperphosphorylation of Cdc25 is the mechanism through which Mos and MAP kinase initiate the activation of Cdc2, or is instead a consequence of Cdc2 activation (Hsiao *et al.*, 1994), remains to be determined.

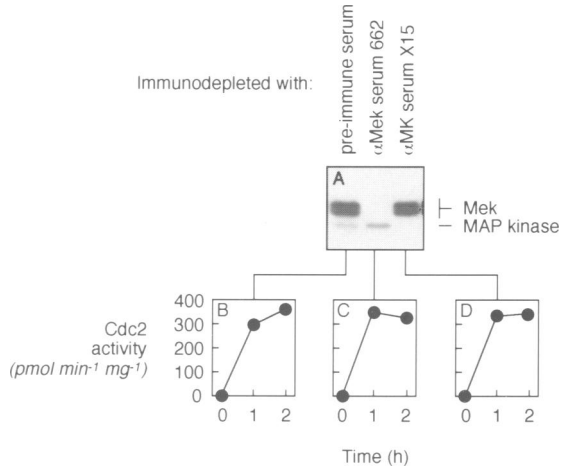

Fig. 3. Activation of Cdc2 by okadaic acid does not require p42 MAP kinase or Mek. Extracts were immunodepleted of their p42 MAP kinase or Mek, or were mock-depleted with pre-immune serum. (**A**) A combined Mek/MAP kinase blot of the depleted extracts. (**B–D**) The response of these three extracts to okadaic acid treatment.

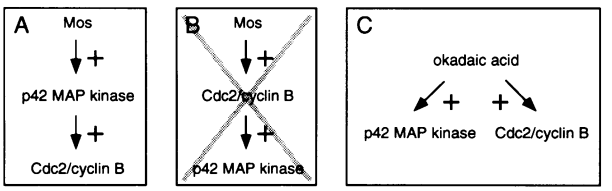

Fig. 4. Schematic view of the activation of Cdc2 by Mos.

Okadaic acid effects

As shown in Figure 1A, H and P, okadaic acid can bring about Cdc2 activation even in extracts that are not supplemented with p42 MAP kinase. The mechanism of okadaic acid-induced Cdc2 activation is poorly understood, but previous studies have reported that okadaic acid-induced Cdc2 activation was not prevented by Mek antibodies (Kosako *et al.*, 1994b), suggesting that p42 MAP kinase is not likely to be involved. We tested this possibility further by preparing extracts whose Mek or p42 MAP kinase had been immunodepleted, and then examining whether okadaic acid could still bring about Cdc2 activation. As shown in Figure 3, either Mek or p42 MAP kinase could be immunodepleted without affecting okadaic acid-induced Cdc2 activation. Thus, okadiac acid can bring about Cdc2 activation in a Mek- and p42 MAP kinase-independent fashion.

Summary and conclusions

In summary, we have reconstituted the link between Mos and Cdc2 in a *Xenopus* oocyte extract system, and have found that Cdc2 activation depends upon the activation of p42 MAP kinase. These results are shown schematically in Figure 4. p42 MAP kinase activation is essential for Mos-induced Cdc2 activation (Figure 4A) and, in agreement with previous results (Nebreda *et al.*, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993), Cdc2 activation is not required for Mos-induced p42 MAP kinase activation (Figure 4B). Our findings raise the possibility that, like Mos, p42 MAP kinase is an important component of the meiotic trigger *in vivo*. The technical advantages of the extract system may allow the delineation

of the pathway through which Mos and p42 MAP kinase regulate Cdc2.

Thiophosphorylated, active MAP kinase has been reported to bring about Cdc2 activation when microinjected into oocytes, but only when protein synthesis is permitted (Haccard *et al.*, 1995). The present work establishes that there can be a more direct connection between p42 MAP kinase and Cdc2, since protein synthesis is not required for Mos- and p42 MAP kinase-dependent Cdc2 activation in extracts. Active Cdc2/cyclin complexes can bring about p42 MAP kinase activation (Gotoh *et al.*, 1991), so that once Cdc2/cyclin B and p42 MAP kinase are sufficiently active, a positive feedback loop could keep them locked in their active states.

Materials and methods

Preparation of oocyte extracts

Xenopus ovarian tissue was obtained surgically. Oocytes were defolliculated with 2 mg/ml collagenase and 0.5 mg/ml polyvinylpyrrolidone in Ca^{2+}-free modified Barth's saline (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.6) for 1.5–2 h at room temperature. The oocytes were washed four times with modified Barth's saline containing 0.41 mM $CaCl_2$, 0.41 mM $Ca(NO_3)_2$, 1 mg/ml bovine serum albumin and 50 μg/ml gentamycin. Stage VI oocytes were sorted manually, incubated at 16°C for at least 8 h, rinsed twice in EB buffer (0.25 M sucrose, 0.1 M NaCl, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride), and twice in this buffer plus 100 μg/ml cytochalasin B. Concentrated oocyte extracts were prepared essentially as previously described (Shibuya *et al.*, 1992). Washed oocytes were spun at low speeds to remove the excess EB buffer and crushed by centrifugation at 12 000 *g* for 15 min at 4°C. Cytoplasmic extracts, which were between lipid and yolk layers, were collected and spun repeatedly until clear lysates were obtained. Aliquots were frozen on dry ice and stored at –80°C.

Recombinant proteins

Purified bacterially expressed malE-Mos proteins were provided by Monica Murakami and George Vande Woude (Yew *et al.*, 1992). Recombinant histidine-tagged rat Erk2 proteins were expressed and purified as described (Robbins *et al.*, 1993). Purified bacterially expressed Mkp-1 was provided by Hong Sun and Nicholas Tonks (Sun *et al.*, 1993).

Kinase assays

Samples were assessed for MAP kinase activity by a myelin basic protein (MBP) kinase assay. Samples were first diluted 30-fold with EB buffer and 1/10 of each sample was incubated with kinase buffer [60 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 5 mM EGTA, 100 μM [γ-^{32}P]ATP (300–600 c.p.m./pmol), 7.5 μM protein kinase A inhibitor peptide, 0.75 mM Na_3VO_4 and 0.4 mg/ml MBP] at 30°C for 15 min. The kinase reaction mixture was spotted on Whatman P81 paper, washed twice with 1% acetic acid and twice with water, and counted in a scintillation counter by Cerenkov radiation.

In several experiments, MAP kinase activity was also assessed a second way, by immunoprecipitating p42 MAP kinase with antiserum X15, washing the immunoprecipitates, and carrying out an immune complex kinase assay in kinase buffer with MBP as a substrate. The products were separated by SDS–PAGE, and incorporation of radiolabel into MBP was detected by autoradiography and quantified by Cerenkov counting. In all cases, trends in the total MBP kinase activity assessed as described above agreed well with trends in the specific p42 MAP kinase activity assessed by immune complex kinase assay.

To assay Cdc2 kinase activity, 9/10 of each sample was immunoprecipitated with $p13^{suc1}$ beads (Upstate Biotechnology Inc.) at 4°C for 4 h. The beads were washed twice with IP buffer (25 mM Tris, pH 8.0, 10 mM $MgCl_2$, 15 mM EGTA, 0.1% Triton X-100, 0.5 mM NaF, 60 mM β-glycerol phosphate, 0.5 mM Na_3VO_4 and 0.1% bovine serum albumin), resuspended in a kinase assay mixture containing 20 mM MOPS, pH 7.2, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 75 μM [γ-^{32}P]ATP (300–600 c.p.m./pmol) and 50 μM histone H1 phosphoacceptor peptide (PKTPKKAKKL; Upstate Biotechnology Inc.), and incubated at 30°C for 15 min. The kinase reaction mixture were spotted on Whatman P81 paper, washed, and counted as described above.

Samples were assessed for Mek activity by a linked Mek/MAP kinase assay. Mek immunoprecipitates were washed twice with IP buffer, and incubated at 30°C with recombinant rat Erk2/p42 MAP kinase (2 μg/ml), ATP (60 μM), HEPES (pH 7.5, 20 mM), β-mercaptoethanol (10 mM), and $MgCl_2$ (20 mM). After 10 min of incubation, 10 μg of MBP and 0.2 μCi [γ-^{32}P]ATP were added, and the reaction mixture was incubated at 30°C for another 5 min. The kinase reaction mixture was spotted on Whatman P81 paper, washed, and quantified as described above.

Immunoblotting

Samples were separated on low-bis polyacrylamide SDS gels (acrylamide:bisacrylamide ratio 100:1) and transferred to Immobilon P (Millipore) blotting membranes. Proteins were detected with Mek antibody 662 (Hsiao *et al.*, 1994), MAP kinase antibodies X15 and DC3 (Hsiao *et al.*, 1994), a polyclonal anti-phosphotyrosine antiserum (Ferrell *et al.*, 1991) or a retro-eluted goat anti-Cdc25 antiserum (a gift from J.Maller), followed by [^{125}I]protein A.

p42 MAP kinase and Mek immunodepletion

p42 MAP kinase was depeleted from extracts by three sequential rounds of immunodepletion with antibody X15, as described (Hsiao *et al.*, 1994). Mek was immunodepleted by a single round of incubation with antibody 662.

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Note added in proof

Gotoh and co-workers recently reported that injection of a MAP kinase phosphatase (CL100/Mkp-1) mRNA into oocytes inhibits progesterone-induced activation of MAP kinase and Cdc2 [Y.Gotoh, N.Masuyama, K.Dell, K.Shirakabe and E.Nishida (1985) Initiation of *Xenopus* oocyte maturation by activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.*, **270**, 25898–25904]. Similarly, we have found that expression of a *Xenopus* CL100 in oocytes inhibits progesterone-induced activation of MAP kinase and Cdc2, whereas expression of comparable levels of a catalytically inactive mutated form of Mkp-1/CL100 does not (C.-Y.F.Huang, M.L.Sohaskey, S.-J.Shih and J.E.Ferrell,Jr, submitted). These findings agree well with the extract studies reported here.