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Mos Induces the In Vitro Activation of Mitogen-activated Protein Kinases in Lysates of Frog Oocytes and Mammalian Somatic Cells

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Mitogen-activated protein kinases (MAPKs) are rapidly and transiently activated when both quiescent Go-arrested cells and G2-arrested oocytes are stimulated to reenter the cell cycle. We previously developed a cell-free system from lysates of quiescent *Xenopus* oocytes that responds to oncogenic H-ras protein by activating a MAPK, p42MAPK. Here, we show that the oncogenic protein kinase mos is also a potent activator of p42MAPK in these lysates. Mos also induces p42MAPK activation in lysates of activated eggs taken at a time when neither mos nor p42MAPK is normally active, showing that the mos-responsive MAPK activation pathway persists beyond the stage where mos normally functions. Similarly, lysates of somatic cells (rabbit reticulocytes) also retain a mos-inducible MAPK activation pathway. The mos-induced activation of MAPKs in all three lysates leads to phosphorylation of the pp90rsk proteins, downstream targets of the MAPK signaling pathway in vivo. The in vitro activation of MAPKs by mos in cell-free systems derived from oocytes and somatic cells suggests that mos contributes to oncogenic transformation by inappropriately inducing the activation of MAPKs.

INTRODUCTION

The oncogene mos encodes a serine/threonine kinase that is expressed in germ cells but is normally undetectable in most somatic cells. Transformation of mammalian somatic cells by mos leads to unrestricted cell proliferation (reviewed by Park and Vande Woude, 1989; Freeman and Donoghue, 1991). Several important insights into the normal physiological role of mos have come from studies using frog and mouse oocytes, where mos is required for normal reentry into the cell cycle and for imposing cell cycle arrest at metaphase of meiosis II (Sagata *et al.*, 1988, 1989b; O'Keefe *et al.*, 1989; Paules *et al.*, 1989; Daar *et al.*, 1991; Kanki and Donoghue, 1991). Fully grown *Xenopus* oocytes are arrested in prophase at the G2/M border of meiosis. On stimulation by their natural mitogen, progesterone, oocytes reenter the cell cycle, proceed through meiosis I, and arrest in metaphase of meiosis II (a process referred to as oocyte maturation); at this point the mature oocytes are called eggs. Fertilization of the egg breaks this second cell cycle arrest and initiates the rapid embryonic mitotic cell cycle (reviewed by Masui and Shibuya, 1987).

Although *Xenopus* oocytes contain a stockpile of maternal mos mRNA, mos protein is undetectable in these cells (Sagata *et al.*, 1988, 1989b; Freeman *et al.*, 1989). Mos synthesis is initiated in response to progesterone; mos protein accumulates during meiosis I, and both mos protein and kinase activity remain high in the metaphase II-arrested egg (Sagata *et al.*, 1989a,b; Watanabe *et al.*, 1989). After fertilization, mos kinase activity drops rapidly; the loss of kinase activity is followed by proteolysis of mos (Sagata *et al.*, 1989b; Watanabe *et al.*, 1989, 1991b).

Newly synthesized mos protein plays an essential role in cell cycle reentry in *Xenopus* oocytes. Ablation of mos mRNA by antisense oligonucleotides in oocytes prevents resumption of the meiotic cell cycle in response to progesterone or insulin (Sagata *et al.*, 1988). Microinjection of mos mRNA or protein into G2-arrested oocytes induces the activation of maturation or M-phase promoting factor, entry into M-phase, and progress through the meiotic cell cycle, providing direct evidence for the ability of mos itself to induce cell cycle reentry (Freeman *et al.*, 1989; Sagata *et al.*, 1989a; Yew *et al.*, 1992). Mos also functions at a second, and very different, point in

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the meiotic cell cycle, where it appears to be a component of cytostatic factor (CSF), an activity that maintains arrest at metaphase II (reviewed by Masui and Shibuya, 1987). Similar to cytoplasm containing CSF activity, injection of mos into rapidly dividing embryonic cells arrests the cell cycle at metaphase (Watanabe *et al.*, 1991a). Furthermore, CSF activity can be depleted from metaphase II-egg cytoplasm with mos antibodies (Sagata *et al.*, 1989b). Thus, mos appears to play important roles in the release from G2-arrest of oocytes and induction of meiotic metaphase arrest in the egg.

Mitogen-activated protein kinases (MAPKs) are rapidly and transiently activated by diverse extracellular signals when quiescent Go-arrested cells are stimulated to reenter the cell cycle (reviewed by Cobb *et al.*, 1991; Posada and Cooper, 1992a). In embryonic systems, MAPKs are activated during oocyte maturation in the sea star (Sanghera *et al.*, 1990), frog (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991b; Posada *et al.*, 1991), and clam (Shibuya *et al.*, 1992a). In *Xenopus* oocytes, cell cycle reentry is accompanied by the one-time activation of a 42-kDa MAPK (p42MAPK). The onset, maintenance, and disappearance of p42MAPK activity is virtually coincidental with that of mos activity. High levels of p42MAPK activity are maintained in the metaphase II-arrested egg until fertilization, when p42MAPK is rapidly inactivated (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991b; Posada *et al.*, 1991). Inactive p42MAPK persists after fertilization but is not detectably reactivated during the early embryonic cell cycles (Ferrell *et al.*, 1991).

MAPKs are activated by phosphorylation of nearby threonine and tyrosine residues by MAPK kinase (MAPKK), a dual specificity kinase that is itself positively regulated by phosphorylation on serine/threonine residues (Anderson *et al.*, 1990; Ahn *et al.*, 1991; Gómez and Cohen, 1991; Crews and Erikson, 1992; Kosako *et al.*, 1992; Nakielny *et al.*, 1992). Genetic studies have revealed the existence of MAPK relatives KSS1/FUS3 in budding yeast and spk-1 in fission yeast whose activities are required for cell cycle arrest and differentiation along the mating pathway (reviewed by Elion *et al.*, 1991). KSS1/FUS3 and spk-1 activities depend on the upstream kinases STE7 and byr-1, respectively, whose activities are in turn regulated by STE11 (for STE7) and byr-2 (for byr-1). Vertebrate MAPKKs show considerable sequence similarities to STE7 and byr-1, but no vertebrate MAPKK kinases (MAPKKKs) resembling STE11 or byr-2 have been identified to date (Crews *et al.*, 1992; Seger *et al.*, 1992; Kosako *et al.*, 1993; Wu *et al.*, 1993). Raf, another ser/thr kinase originally identified as an oncogene (Rapp *et al.*, 1983), can phosphorylate and reactivate phosphatase-inactivated MAPKK in vitro using purified components (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Mos can substitute for raf in a similar assay (Posada *et al.*, 1993). However, mos and raf bear little resemblance to STE11 or byr-2, and limitations of the in vitro assay system leave open the possibility that in vivo mos and/or raf are upstream activators of a yet to be identified MAPKKK.

Whereas the earliest steps in the various mitogen-stimulated MAPK activation pathways differ, virtually all of these signaling pathways converge through ras and proceed on to MAPK via a kinase cascade (reviewed by Ruderman, 1993). Injection of oncogenic H-ras protein into *Xenopus* oocytes induces the activation of p42MAPK and cell cycle reentry (Birchmeier *et al.*, 1985; Hattori *et al.*, 1992; Pomerance *et al.*, 1992; Shibuya *et al.*, 1992a). We and others have recently established cell-free systems from quiescent G2-arrested *Xenopus* oocytes that respond to ras addition by activating p42MAPK in vitro (Hattori *et al.*, 1992; Shibuya *et al.*, 1992b). In this study, we show that mos is a more potent activator of p42MAPK in the cell-free system than ras. Addition of recombinant *Xenopus* c-mos protein to concentrated cytoplasmic lysates of *Xenopus* oocytes triggers the rapid activation of p42MAPK. Mos also induces the rapid and complete in vitro activation of p42MAPK in lysates of postmeiotic eggs, in which endogenous mos has been degraded and p42MAPK is normally inactive. Lysates of mammalian somatic cells (reticulocytes) also respond to mos addition by the activation of two MAPKs, p44 (ERK1) and p42 (ERK2). In all three types of lysates, MAPKs are maintained in an activated state by mos, suggesting that mos contributes to the transformation of somatic cells by inappropriately inducing MAPK activation in the absence of any extracellular signals.

MATERIALS AND METHODS

Purification of Recombinant Proteins

Recombinant *Xenopus* mos proteins with N-terminal maltose-binding protein epitopes were produced in *Escherichia coli* (TB1 strain) harboring constructs pMALcRI-Xe and pMALcRI-XeKM and purified by affinity chromatography as previously described (Yew *et al.*, 1992). The two fusion proteins used contained either the wild-type mos (mos^{wt}) or a mutant inactive form in which lysine 90 in the ATP-binding site was changed to arginine (mos^{km}). Briefly, proteins were expressed, and after sonication of the cells, the soluble supernatant was mixed with amylose-Sepharose CL-4B resin (New England Biolabs, Beverly, MA). After extensive washing, the mos proteins were eluted from the resin with 10 mM maltose, 88 mM NaCl, and 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 6.8, and then dialyzed against the same buffer without maltose. The final mos^{wt} and mos^{km} protein preparations had the same degree of purity and were adjusted to the same concentrations with Centricon-10 concentrators (Amicon, Dancers, MA) before storage in aliquots at −80°C. Three separately made mos preparations gave similar results.

Oligo-histidine-tagged recombinant p42MAPK protein (^{His}ERK2) and oncogenic H-ras^{val12} ($^{His}ras^{val12}$) were prepared as described (Shibuya *et al.*, 1992b). The expressed proteins in the supernatants of bacterial lysates were bound to affinity resin comprised of a Ni^{2+}-charged nitrilotriacetic acid (Ni-NTA) ligand attached to Sepharose CL-6B (Qiagen, Chatsworth, CA). After washing, bound proteins were batch eluted with increasing concentrations of imidazole, dialyzed, concentrated, and stored in aliquots at −80°C.

Frog Oocyte and Egg Lysates

Oocyte and egg lysates were prepared as described (Shibuya *et al.*, 1992b), modified from previous procedures (Wasserman and Masui,

1976; Lohka and Maller, 1985; Murray and Kirschner, 1989). Briefly, late stage V and VI oocytes (≥1.2 mm) (Dumont, 1972) were defolliculated and isolated from ovaries of unprimed frogs by collagenase treatment (Krohne and Franke, 1983). After rinsing in extraction buffer (EB) of 0.25 M sucrose, 0.1 M NaCl, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2, 10 μg/ml each of leupeptin, pepstatin, and chymostatin at 4°C, oocytes were crushed by centrifugation at 15 000 × *g* for 15 min. The supernatant was collected and cytochalasin B was added (50 μg/ml) before recentrifugation. Aliquots (100 μl) of the final supernatant were frozen in liquid nitrogen and stored at −80°C.

Lysates of ovulated, dejellied, metaphase II-arrested eggs were prepared in the same way, except that the EB included 10 mM NaF (recrystallized) (Sigma, St. Louis, MO) and 10 mM ethylene glycolbis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). Interphase-arrested lysates were prepared from dejellied unfertilized eggs that had been incubated in the protein synthesis inhibitor cycloheximide (100 μg/ml) in 20% modified Steinberg's solution (Masui, 1967) for 15 min before activation by electric shock. Incubation was continued in this medium for 45 min before making lysates with EB. Oocyte and egg lysates had protein concentrations of 40–50 mg/ml measured using an assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard (Bradford, 1976).

Reticulocyte Lysates and In Vitro Translation

The mammalian somatic cell lysate used was nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI). Several different batches of lysate responded similarly in the MAPK activation assays. *Xenopus* c-mos mRNA was prepared by in vitro transcription of the plasmid according to Sagata *et al.* (1989a). mRNA preparations were heated at 65°C for 10 min, diluted in RNAse-free DH_2O, and added to reticulocyte lysates on ice along with ^{35}S-methionine (1.0 mCi/ml final concentration) (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Translations were done at 30°C, and samples were taken for analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE 1 vol translation mix/10 vol sample buffer) (Laemmli, 1970) followed by immunoblotting or autoradiography.

In Vitro Assays of MAPK Activation

In vitro assays of MAPK activation were done as previously described (Shibuya *et al.*, 1992b). Assay samples (20–50 μl) were prepared on ice by mixing 16 vol oocyte or egg lysate, 1 vol of an ATP-regenerating system (20 mM ATP, 20 mM $MgCl_2$, 0.2 M creatine phosphate, and 1 mg/ml creatine kinase [Boehringer Mannheim, Indianapolis, IN] in EB), and 3 vol of EB or other additions. Reticulocyte lysate assays were done using the same proportions of lysate, ATP-regenerating system, and other additions. For assay of mos^{wt} and mos^{km}, proteins were added to assay samples to obtain final concentrations ranging from 15 to 45 μg/ml. Assay of ras and okadaic acid were as previously described (Shibuya *et al.*, 1992b). $^{His}ras^{val12}$ protein was loaded with GTP and added to assay samples at final concentrations of 125 μg/ml. Okadaic acid was added to a final concentration of 1.0 μM in samples from a stock solution of 50 μM in 10% dimethyl sulfoxide. During the assay, samples were incubated at 21–23°C, and aliquots were taken at intervals for immunoblotting (1 vol lysate mix/10 vol sample buffer) or kinase assays (1 vol lysate mix/10 vol EB with 10 mM NaF, 0.2 mM Na_3VO_4).

Immunoblotting

Immunoblotting with MAPK antiserum (anti-ERK1 691) (Boulton and Cobb, 1991) and phosphotyrosine antibodies (Druker *et al.*, 1989) was as previously described (Shibuya *et al.*, 1992a). Two anti-pp90rsk antisera (a generous gift of J. Blenis, Harvard Medical School) were raised against either the full-length recombinant chicken pp90rsk or a synthetic peptide corresponding to the C-terminal 20 amino acids of the mouse pp90rsk (Alcorta *et al.*, 1989; Chen and Blenis, 1990; Blenis, unpublished data). The pp90rsk antisera were diluted 1:1000 in primary antibody solution (Shibuya *et al.*, 1992a), and blots of 10% polyacrylamide gels were incubated overnight. Detection of primary antibody binding was by alkaline phosphatase-conjugated secondary antibody following the manufacturer's instructions (Promega).

Kinase Assays

To measure activation of MAPKK, we have developed an assay in which inactive recombinant mammalian ^{His}ERK2 is incubated in the lysates at a concentration of 100 μg/ml and then retrieved and purified by binding to Ni-NTA affinity resin (Qiagen). The bound protein is then assayed for kinase activity using myelin basic protein (MBP) as a substrate and for the presence of phosphotyrosine in the electrophoretically shifted protein by immunoblotting (Shibuya *et al.*, 1992b). The increase in ^{His}ERK2 kinase activity indicates activation of MAPKK.

To determine cdc2 activity, 1 vol of lysate sample was diluted with 9 vol of EB that had been supplemented with 10 mM NaF and 1 mM Na_3VO_4. Five microliters of the diluted sample was assayed for kinase activity using histone H1 (Boehringer-Mannheim) as a substrate (Shibuya *et al.*, 1992a). H1 or MBP bands were excised from dried gels and immersed in Aquasol (DuPont, Wilmington, DE); ^{32}P-incorporation was quantified in a scintillation counter.

RESULTS

Mos Induces the Activation of p42MAPK in Quiescent Frog Oocyte Lysates

Full-length recombinant *Xenopus* c-mos protein (mos^{wt}) and a mutant kinase-inactive form (mos^{km}), each with an N-terminal maltose-binding protein epitope, were purified by affinity-chromatography (Yew *et al.*, 1992) and tested for their ability to induce MAPK activation in vitro. When mos^{wt} was added to lysates of quiescent *Xenopus* oocytes, a portion of the p42MAPK was rapidly shifted to an electrophoretically retarded tyrosine-phosphorylated form within 30 min of addition, and by 1 h all the p42MAPK was shifted to the tyrosine-phosphorylated slower mobility form (Figure 1A) that, as shown below and elsewhere, represents the activated form of the kinase (Anderson *et al.*, 1990; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a; Posada and Cooper, 1992b; Shibuya *et al.*, 1992b). In contrast, when an equal amount of inactive mos^{km} was added to lysates, there was no change in the mobility or tyrosine phosphorylation of p42MAPK (Figure 1B). Higher concentrations of mos (up to 45 μg/ml) in the lysates did not induce a more rapid activation of p42MAPK. p42MAPK activation by mos did not require protein synthesis, because comparable activation was achieved in the presence of 100 μg/ml cycloheximide.

To directly and specifically test for the induction of MAPKK activity, inactive recombinant oligohistidine-tagged mammalian p42MAPK protein (^{His}ERK2) was added to the lysates with or without mos, retrieved and purified by binding to Ni-NTA affinity beads, and then assayed for kinase activity toward MBP as described previously (Shibuya *et al.*, 1992b). The addition of mos led to the appearance of MAPKK activity, as determined by the tyrosine phosphorylation of the recovered

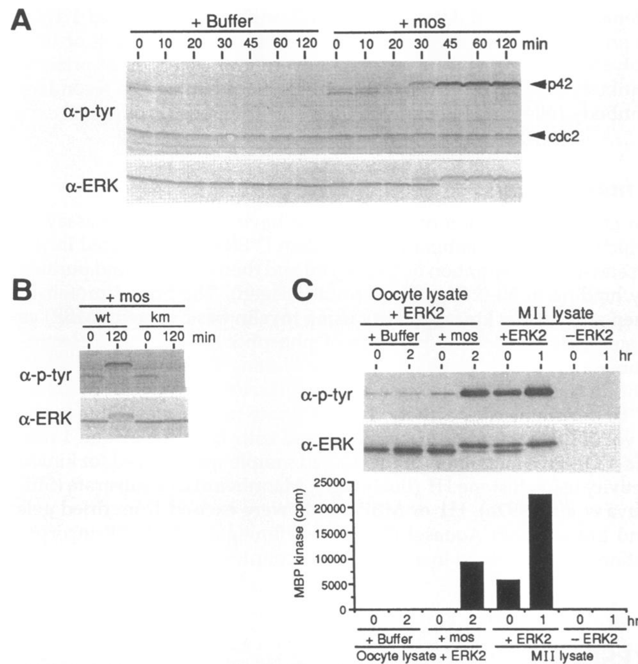

Figure 1. Recombinant *Xenopus* mos protein induces the activation of p42MAPK in lysates of quiescent *Xenopus* oocytes. (A) Extraction buffer or mos protein (final concentration 15 μg/ml) was added to oocyte lysates with an ATP-regenerating system, and samples were incubated at 21°C. Aliquots were taken at the indicated intervals and analyzed by immunoblotting with anti-phosphotyrosine antibody (α-p-tyr) and anti-ERK1 antibody (α-ERK). (B) Mos^{wt} or mos^{km} (30 μg/ml) were added to oocyte lysates and analyzed by immunoblotting as in A. (C) Recombinant mammalian ^{His}ERK2 was incubated in oocyte lysates with or without mos protein (30 μg/ml) and in unactivated egg lysates (MII) for the times indicated. The epitope-tagged protein was then retrieved with affinity resin, washed, and assayed for kinase activity using MBP as a substrate.

^{His}ERK2, and its increased ability to phosphorylate MBP (Figure 1C). In this experiment, mos was able to induce about 40% of the highest levels of MAPKK activity found in lysates of unfertilized eggs, which rises during meiosis I and stays high in the metaphase II-arrested unfertilized egg. These results demonstrate directly that mos can function as a strong positive regulator of the MAPK activation pathway in oocytes, which are the only cells to date in which mos is known to be required for physiologically normal cell cycle reentry.

Although mos Protein Is Lost After Fertilization, the mos-inducible p42MAPK Activation Pathway Is Retained

During the normal course of oocyte maturation, mos activity appears in response to progesterone, rises during meiosis I and meiosis II, and is kept high in the metaphase II-arrested egg. After fertilization of the egg, mos is rapidly inactivated and degraded within 30 min, and no new mos synthesis is detected during the early mitotic cell cycles (Sagata *et al.*, 1988, 1989a,b; Watanabe *et al.*, 1989, 1991b). Similarly, p42MAPK activity appears as oocytes resume the cell cycle, stays high during meiosis I and II, and disappears after the metaphase II egg is fertilized; its reappearance is not detectable during the subsequent mitotic cell cycles (Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a; Posada *et al.*, 1991). Thus, in this system both mos and p42MAPK are normally active only during oocyte maturation. Unlike mos, however, the inactive form of p42MAPK persists throughout the early cell cycles and can be reactivated, at least in vitro, by various agents, including oncogenic ras protein and cyclin-activated cdc2 (Shibuya *et al.*, 1992b). It was, therefore, of interest to determine if lysates made from eggs collected after mos disappearance and p42MAPK inactivation still retained the mos-responsive pathway leading to p42MAPK activation. To answer this question, we prepared lysates from eggs that had been activated by electric shock (to mimic fertilization) and collected in interphase of the first mitotic cell cycle. When mos^{wt} was added to these interphase lysates, p42MAPK activation was as rapid and complete (Figure 2) as in mos-stimulated oocyte lysates (Figure 1A).

These results demonstrate that 1) the mos-inducible p42MAPK activation pathway is preserved in eggs even after mos disappearance and 2) this pathway responds to exogenous mos as rapidly and effectively as the one in oocytes. Thus, in this system, the inappropriate presence of mos can lead to the inappropriate reappearance of active p42MAPK.

Mos-stimulated Activation of p42MAPK in Oocyte Lysates Occurs Independently of cdc2 Activation

Injections of recombinant mos protein or mRNA into quiescent oocytes can induce all the events of meiotic maturation, including p42MAPK activation, cyclinB/cdc2 kinase activation, completion of meiosis I, and arrest at metaphase of meiosis II (Freeman *et al.*, 1989; Sagata *et al.*, 1989a; Yew *et al.*, 1992; Posada *et al.*, 1993). To ask if mos also led to the in vitro activation of cdc2, mos was added to an oocyte lysate and cdc2 activation was monitored indirectly by the loss of tyrosine phosphorylation (on phosphotyrosine immunoblots) and di-

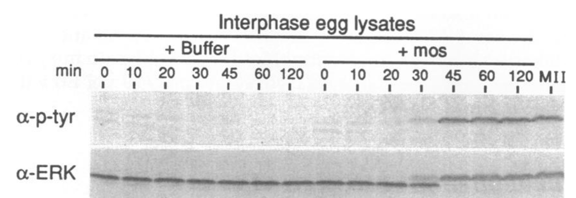

Figure 2. Lysates made from interphase-arrested eggs retain the ability to activate p42MAPK in response to mos. Extraction buffer or mos protein (30 μg/ml) was added to interphase-arrested egg lysates. Samples were taken at the indicated times during incubation at 21°C and analyzed by immunoblotting as in Figure 1A.

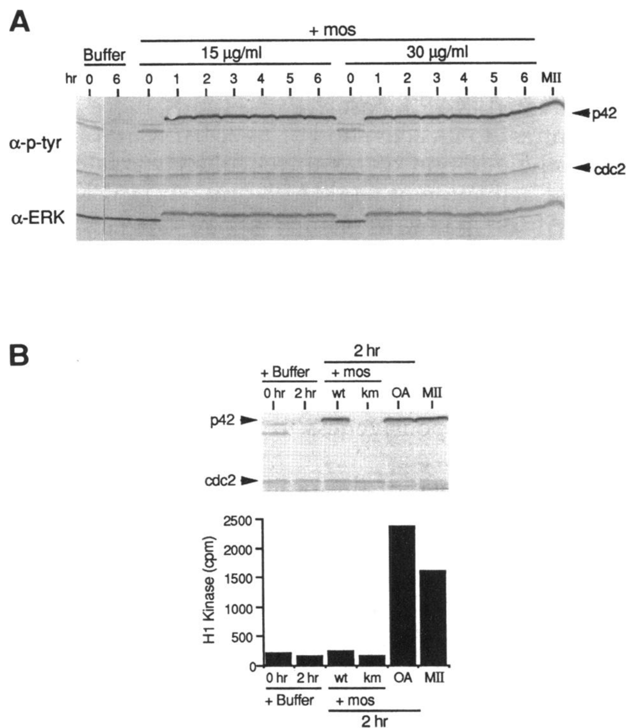

Figure 3. Mos activates p42MAPK in oocyte lysates by a cdc2-independent pathway. (A) Fifteen or 30 μg/ml mos was added to oocyte lysates and incubated at 21°C, and at 1 h intervals, samples were analyzed by immunoblotting with α-p-tyr and α-ERK antibodies. (B) Similar samples as in A were immunoblotted with α-p-tyr antibodies at 0 and 2 h of incubation. Duplicate samples were assayed for activation of cdc2 using histone H1 as a substrate.

rectly by the appearance of histone H1 kinase activity. Whereas p42MAPK was fully activated within 1 h of mos addition, neither the loss of the inhibitory tyrosine phosphorylations on cdc2 nor the appearance of H1 kinase activity was seen as late as 6 h after mos addition (Figure 3). The mos protein was stable throughout the incubation (determined by immunoblotting) probably because of the N-terminal MBP epitope, which may prevent the ubiquitin-mediated degradation of mos seen by others in vivo (Nishizawa *et al.*, 1992; Okazaki *et al.*, 1992). This result establishes two points. First, mos can activate p42MAPK by a pathway that does not require cdc2. Second, because the endogenous cyclin B/cdc2 complexes in oocyte lysates can be activated perfectly well in response to the addition of other reagents such as cyclin A or B or the phosphatase inhibitor okadaic acid (Figure 3B) (Shibuya *et al.*, 1992b), the oocyte lysates described here must lack an activity that is necessary to couple mos to the cdc2 activation pathway. It is noteworthy that ras also triggers activation of both pathways in vivo (Birchmeier *et al.*, 1985; Allende *et al.*, 1988; Pomerance *et al.*, 1992; Shibuya *et al.*, 1992b) but only the p42MAPK activation pathway in vitro (Shibuya *et al.*, 1992b), a result that suggests that the mos- and ras-activated pathways share common elements.

pp90rsks Are Phosphorylated in Oocyte Lysates in Response to ras and mos-Activation of p42MAPK

One of the earliest and best-characterized targets of MAPK are the 90-kDa ribosomal S6 kinases, or pp90rsks (reviewed by Erikson, 1991). To determine if the p42MAPK signaling pathway extended in vitro to the pp90rsks, we immunoblotted oocyte lysates stimulated by either mos or ras with pp90rsk antibodies. The mouse pp90rsk antibodies recognized two proteins of 80 and 82 kDa in lysates of quiescent oocytes. The addition of either ras^{val12} or mos^{wt} to these lysates led to progressive retardations in the pp90rsk mobilities to 91 and 93 kDa (Figure 4, top). Temporally, the mobility shift of the pp90rsks closely followed the activation of p42MAPK in each case (Figure 4, bottom). The same changes were seen with the chicken pp90rsk antibodies. The increased size of the pp90rsks is comparable with the highly phosphorylated fully activated pp90rsks purified from unfertilized *Xenopus* eggs (Erikson and Maller, 1986, 1989; Erikson *et al.*, 1987; Jones *et al.*, 1988). Therefore, it is likely that the mobility-shifted pp90rsks represent the phosphorylated active forms of these kinases. These results indicate that kinase cascades triggered by ras and mos proceed through MAPKK and p42MAPK and extend, at least, to pp90rsks.

Mos, but not ras, Induces the Activation of p42MAPK and p44MAPK in Lysates of Rabbit Reticulocytes

Finding that both mos and ras could induce the nonphysiological reappearance of p42MAPK activity in lysates of interphase-arrested eggs, we asked if mos could induce MAPK activation in a lysate from mammalian somatic cells, rabbit reticulocytes. These cells, which have expelled their nuclei and exited from the cell division cycle, are nearing terminal differentiation as erythrocytes. Reticulocyte lysates have been useful in studying complex biochemical pathways, such as ubiquitin-mediated proteolysis (Bercovich *et al.*, 1989; Scheffner *et al.*, 1990; Ciechanover *et al.*, 1991), and

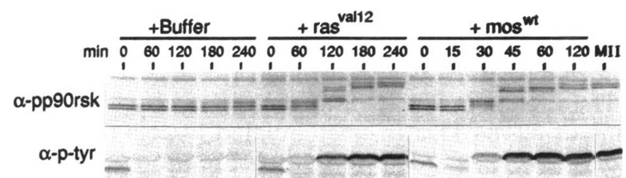

Figure 4. pp90rsk proteins are phosphorylated in oocyte lysates in response to ras and mos activation of p42MAPK. Buffer, $^{His}ras^{val12}$, or mos^{wt} were added to oocyte lysates and samples were analyzed by immunoblotting with α-mouse pp90rsk antibodies and α-p-tyr antibodies.

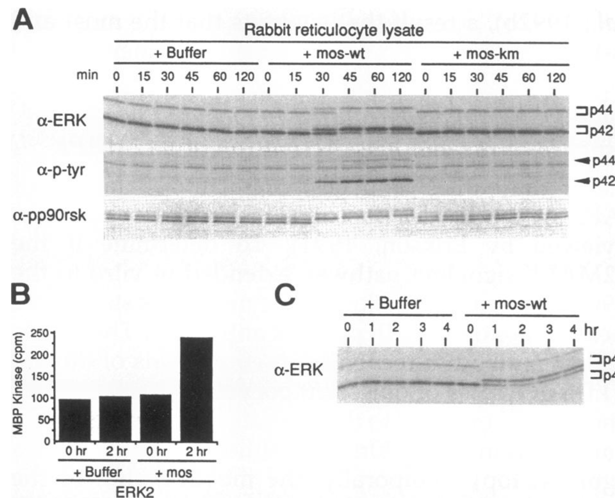

Figure 5. Mos induces the activation of p44MAPK, p42MAPK, and pp90rsk phosphorylation in rabbit reticulocyte lysates. (A) Buffer, mos^{wt}, or mos^{km} were added to rabbit reticulocyte lysates, and samples were taken at intervals during incubation at 21°C followed by analysis by immunoblotting with α-ERK (top), α-p-tyr (middle), and α-mouse pp90rsk (bottom) antibodies. (B) ^{His}ERK2 was added to reticulocyte lysates with or without mos^{wt} and incubated for 0 or 2 h. The tagged protein was then retrieved by binding to affinity resin, washed, and assayed for kinase activity using MBP as a substrate. (C) Buffer or mos^{wt} was added to reticulocyte lysates. Then samples were taken at 1 h intervals and analyzed by immunoblotting with α-ERK antibodies.

commercially available nuclease-treated reticulocyte lysates are widely used for the in vitro synthesis of proteins from exogenous mRNAs (Jackson and Hunt, 1983). Reticulocyte lysates contained two proteins (Figure 5A) that correspond to p44MAPK (ERK1) and p42MAPK (ERK2) in numerous other mammalian tissues (reviewed by Cobb *et al.*, 1991). The addition of mos^{wt}, but not mos^{km}, to these lysates led to the upward shift and tyrosine phosphorylation of both p44MAPK and p42MAPK (Figure 5A, top and middle). The changes in the MAPKs were first detected by 30 min of incubation and were maintained for at least 2 h. Just as in the *Xenopus* oocyte and egg lysates, mos led to the activation of MAPKs by inducing the activation of MAPKK activity (Figure 5B). Unlike the transient activation of p44MAPK and p42MAPK seen in other mammalian cell types in vivo, the addition of mos to reticulocyte lysates appears to reproduce the rapid activation of MAPKs but not their inactivation (Figure 5C), suggesting that high levels of mos may irreversibly activate MAPKs.

The mouse pp90rsk antibodies recognized two proteins of 82–84 kDa in unstimulated lysates that were progressively retarded in their electrophoretically mobility as the MAPKs were activated, stabilizing at 85–87 kDa (Figure 5A, bottom). The same proteins were also detected using the chicken pp90rsk antibodies. Similar shifts in electrophoretic mobility were seen in phosphorylated activated pp90rsks from serum-stimulated chicken, mouse, and human cells (Chen and Blenis, 1990), suggesting that the reticulocyte pp90rsks were phosphorylated and activated in response to the mos-activated MAP kinases.

In contrast to its activation of p42MAPK in *Xenopus* lysates, the addition of ras to reticulocyte lysates did not cause activation of p44MAPK and p42MAPK (Figure 6). At this point, we cannot say whether the lack of ras responsiveness in reticulocyte lysates is because of inability to process the exogenous ras or couple ras to downstream targets. Therefore, although reticulocyte lysate is valuable as a mammalian somatic cell lysate that has an activatable MAPK signaling pathway, the *Xenopus* oocyte lysate may be more generally useful because of its responsiveness to a wider range of mitogenic stimuli.

Taking advantage of the protein synthetic capacity of reticulocyte lysates, we asked if mos mRNA would lead to MAPK activation. The activation of both MAPKs was induced by the addition of mos mRNA (Figure 7A) in a dose-dependent manner (Figure 7B). Translation of similar amounts of proteins from other mRNAs did not activate either MAPK. These results demonstrate that reticulocyte lysates may provide a quick and efficient way to screen a wide variety of cloned genes for potential roles in the MAPK signaling pathway, an approach that avoids laborious and frequently unsuccessful attempts to produce active, recombinant, bacterially expressed proteins.

DISCUSSION

Mos Activates the MAPK Signaling Pathways in Lysates of Frog Oocytes, Eggs, and Mammalian Somatic Cells

We have recently shown that oncogenic H-ras protein can induce the activation of p42MAPK in lysates of *Xenopus* oocytes and eggs (Shibuya *et al.*, 1992b). We show here that another oncogenic protein, *Xenopus* c-mos, is also a potent inducer of p42MAPK activation in vitro. Whereas mos leads to the activation of cdc2 and cell cycle reentry after injection into intact oocytes, mos does not lead to cdc2 activation in oocyte lysates, indicating that mos-induced p42MAPK activation proceeds independently of cdc2. Similar results have recently been obtained by Nebreda and Hunt (1993). Mos

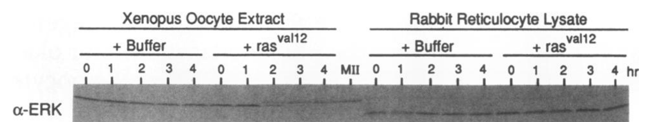

Figure 6. Oncogenic H-ras does not activate MAPKs in reticulocyte lysates. The same preparation of $^{His}ras^{val12}$ was added to a final concentration of 125 μg/ml to either *Xenopus* oocyte lysate or rabbit reticulocyte lysate, and samples taken at intervals during incubation at 21°C were analyzed by immunoblotting with α-ERK antibodies.

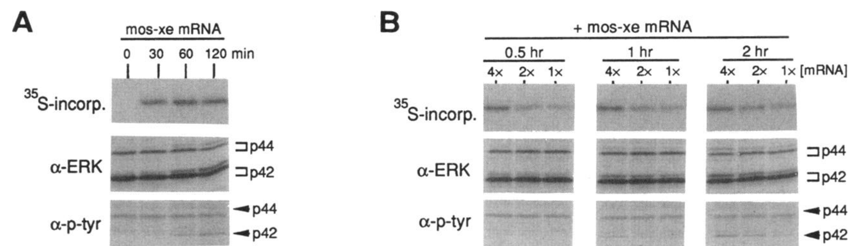

Figure 7. *Xenopus* mos translated from mRNA induces p44MAPK and p42MAPK activation in rabbit reticulocyte lysates. (A) *Xenopus* mos mRNA was added to rabbit reticulocyte lysates with ^{35}S-methionine, and samples were taken during incubation at 30°C. Samples were analyzed for synthesized protein by gel electrophoresis followed by autoradiography and for MAPK activation by immunoblotting with α-ERK and α-p-tyr antibodies. (B) Decreasing amounts of *Xenopus* mos mRNA were added to reticulocyte lysates with ^{35}S-methionine and samples were analyzed as in A.

addition to lysates made from postmeiotic eggs, which lack both mos activity and protein and have only inactive p42MAPK, leads to the reactivation of p42MAPK that is as rapid and complete as that stimulated in oocyte lysates. Somatic cell lysates made from rabbit reticulocytes also retain a mos-inducible MAPK activation pathway. In all three kinds of lysates, the mos-induced activation of MAPKs is followed by phosphorylation of the pp90rsks, two downstream targets of MAPKs in vivo.

cyte lysates probably reflects the fact that these cells are in a physiologically arrested state awaiting appropriate cues to re-enter the cell cycle. The addition of mos protein to these lysates substitutes for mos synthesis, which is required for the initiation and completion of oocyte maturation in vivo (see INTRODUCTION). However, it might not have been predicted that lysates from postmeiotic eggs should retain a mos-responsive p42MAPK activation pathway, because 1) mos is inactivated and degraded shortly after meiosis, 2) the physiological requirement for mos in the initiation and completion of oocyte maturation has passed, and 3) p42MAPK activity does not reappear during the early cleavage division cycles in vivo (Sagata *et al.*, 1989b; Watanabe *et al.*, 1989, 1991b; Ferrell *et al.*, 1991). The ability of mos to trigger the inappropriate activation of p42MAPK could explain its oncogenic effect on somatic cells in which mos is never normally expressed.

Mos Induces the Activation of p42MAPK in Oocyte Lysates Without Activating cdc2

Microinjection of oncogenic ras or mos protein into intact oocytes leads to activation of both p42MAPK and cdc2 (Birchmeier *et al.*, 1985; Hattori *et al.*, 1992; Pomerance *et al.*, 1992; Shibuya *et al.*, 1992b; Yew *et al.*, 1992; Posada *et al.*, 1993). In cell-free systems, however, mos and ras lead to activation of MAPK but not cdc2 (Shibuya *et al.*, 1992b; Nebreda and Hunt, 1993). These findings establish two points. First, although p42MAPK and cdc2 are activated at about the same time in vivo and p42MAPK can be activated experimentally in vitro by activating cdc2, p42MAPK is activated by ras and mos by pathways that clearly are independent of cdc2. Second, these oocyte lysates either lack activities that link ras and mos to cdc2 activation or require changes in existing cytoplasmic conditions to provide this link to cdc2. These changes may be induced in vivo by progesterone stimulation of additional divergent pathways. For example, progesterone does not induce oocyte maturation when protein synthesis is inhibited (Drury and Schorderet-Slatkine, 1975; Wasserman and Masui, 1975), but it does induce some cytoplasmic changes that increase the effectiveness of mos in triggering cdc2 activation and cell cycle reentry (Yew *et al.*, 1992).

Where Does Mos Fit in the MAPK Activation Cascade?

The ability of all three mos-stimulated lysates to activate an exogenous recombinant MAPK suggests that mos leads to MAPK activation by activating MAPKK or a component further upstream in the activation pathway. Does mos function as a MAP kinase kinase kinase (MAPKKK) or does it act further upstream? Posada *et al.* (1993) reported that mos protein can phosphorylate and reactivate phosphatase-inactivated MAPKK in an in vitro assay, providing support for the idea that mos functions as a MAPKKK. However, mos is not the only protein capable of activating MAPKK in vitro. Immunoprecipitates or other highly purified preparations containing the related serine/threonine kinase raf can also phosphorylate and reactivate MAPKK in vitro (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Furthermore, as pointed out in those studies, all the assays included one or more components that could have contained additional kinases. It is possible that in lysates, mos, like ras, is an upstream activator of some yet to be identified MAP kinase kinase kinase kinase in the MAPK activation cascade.

Mos-responsive Lysates Must Contain a Mos Activator/Kinase

Because recombinant mos protein is inactive as purified from bacterial lysates, it must be activated by a com-

ponent that is already present in the lysates. Consistent with this is the finding that recombinant mos acquires the ability to autophosphorylate only after injection into oocytes (Yew *et al.*, 1992). Although the nature of the "mos activator" is unknown, the observations that a portion of the mos^{wt} retrieved after incubation in oocyte lysates is electrophoretically retarded (Shibuya, unpublished data) and the phosphorylation of endogenous mos results in a similarly retarded form (Sagata *et al.*, 1989b; Nishizawa *et al.*, 1992), suggest that the mos activator is a kinase. However, other work suggests that mos activation is not regulated by phosphorylation (Freeman *et al.*, 1992). Whatever its nature, this mos-activating capability is preserved in lysates made from G2-arrested oocytes, from activated eggs after the endogenous mos has been destroyed, and in reticulocyte lysates. Therefore, all three lysates retain both a mos-activating pathway and a mos-responsive pathway. Providing newly synthesized or exogenous mos couples the two pathways. The presence of a mos activator in cells that lack mos could be explained if its normal function was to activate other regulators of signal transduction pathways.

Mos-Inducible MAPK Activation in Cell Proliferation Versus Differentiation

Rabbit reticulocyte lysates also contain a mos-responsive MAPK activation pathway. However, unlike Go-arrested mammalian cells or G2-arrested oocytes that are poised to reenter the cell cycle, reticulocytes are near the end of their differentiation program, in which irreversible events such as nuclear expulsion and mRNA degradation have occurred (reviewed by Rifkind, 1974; Lodish and Small, 1976). It is of particular interest then that these cells retain the ability to respond to mos by activating MAPKs.

V-mos leads to malignant transformation of many cell types, including NIH/3T3 cells and normal rat kidney cells (Kurata *et al.*, 1987 and references therein). However in monocytes, v-mos stops proliferation and induces the phagocytic behaviour, rosette formation, and cell surface antigens characteristic of fully differentiated cells (Kurata *et al.*, 1989). Continuous expression of mos is required to maintain these differentiated features. Thus, mos can induce either cell proliferation or differentiation depending on the cells into which it is introduced. This property is reminiscent of the effects of ras on different cell types. First identified as an oncogene in many cell types (reviewed by Barbacid, 1987; Bollag and McCormick, 1991), ras can promote differentiation in other cell types. For example, in rat phaeochromocytoma (PC12) cells, exposure to nerve growth factor leads to cessation of cell proliferation and induction of differentiation. These changes are mediated by endogenous ras (Thomas *et al.*, 1992; Wood *et al.*, 1992), and introduction of oncogenic ras alone can induce many of these changes (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). It is noteworthy that some of these changes in PC12 cells can also be induced by v-mos (Noda *et al.*, 1985), which now may be seen as a component of a signal transduction pathway.

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