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Mos Stimulates MAP Kinase in *Xenopus* Oocytes and Activates a MAP Kinase Kinase In Vitro

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Several protein kinases, including Mos, maturation-promoting factor (MPF), mitogen-activated protein (MAP) kinase, and MAP kinase kinase (MAPKK), are activated when *Xenopus* oocytes enter meiosis. De novo synthesis of the Mos protein is required for progesterone-induced meiotic maturation. Recently, bacterially synthesized maltose-binding protein (MBP)-Mos fusion protein was shown to be sufficient to initiate meiosis I and MPF activation in fully grown oocytes in the absence of protein synthesis. Here we show that MAP kinase is rapidly phosphorylated and activated following injection of wild-type, but not kinase-inactive mutant, MBP-Mos into fully grown oocytes. MAP kinase activation by MBP-Mos occurs within 20 min, much more rapidly than in progesterone-treated oocytes. The MBP-Mos fusion protein also activates MPF, but MPF activation does not occur until approximately 2 h after injection. Extracts from oocytes injected with wild-type but not kinase-inactive MBP-Mos contain an activity that can phosphorylate MAP kinase, suggesting that Mos directly or indirectly activates a MAPKK. Furthermore, activated MBP-Mos fusion protein is able to phosphorylate and activate a purified, phosphatase-treated, rabbit muscle MAPKK in vitro. Thus, in oocytes, Mos is an upstream activator of MAP kinase which may function through direct phosphorylation of MAPKK.

Fully grown, stage 6 *Xenopus* oocytes are naturally arrested in prophase of meiosis I until progesterone, secreted from surrounding follicle cells, induces progression through meiosis and the production of a mature, unfertilized egg. Following completion of germinal vesicle breakdown (GVBD) and meiosis I, oocytes enter meiosis II but arrest in metaphase awaiting fertilization. During oocyte maturation, a dramatic increase in protein phosphorylation occurs because of activation of a cascade of protein kinases.

Completion of meiosis I and arrest in meiosis II is regulated in part by maturation-promoting factor (MPF), a multisubunit complex consisting of the serine/threonine kinase $p34^{cdc2}$ and cyclin B. In oocytes, MPF is maintained in an inactive state by inhibitory phosphorylations and is referred to as pre-MPF (14–16). Progesterone initiates a cascade of events that creates MPF from pre-MPF. This cascade depends on the synthesis of the Mos protein (19, 41, 53).

The Mos protein is a germ-cell-specific serine/threonine kinase that is absent from immature oocytes and is synthesized from stored mRNA in response to progesterone (41). Translation of Mos is necessary for progesterone- and insulin-induced maturation of oocytes, since injection of Mos antisense oligonucleotides blocks GVBD (41, 42). Mos is not required, however, if maturation is induced by injection of the Ras oncoprotein, even though the Mos-dependent insulin pathway apparently functions through endogenous Ras (8, 13, 27). The Mos protein alone can initiate meiosis, as shown by injecting Mos RNA or a maltose-binding protein (MBP)-Mos fusion protein into oocytes in the absence of any added hormone (41, 53). Kinase-inactive mutant Mos RNA or protein is not able to induce GVBD (19, 53). Mos is the only protein that must be synthesized to initiate maturation, because injection of MBP-Mos protein into cycloheximide-treated oocytes induces GVBD and entry into meiosis I. However, cycloheximide blocks the entry into meiosis II, suggesting that although Mos kinase activity can activate MPF and trigger GVBD, a second protein is needed for the oocytes to proceed to meiosis II and metaphase arrest (53).

Mos is also responsible, in part, for the stability of MPF in the unfertilized egg (43, 53). Fertilization brings about the degradation of cyclin B and a precipitous drop in MPF activity, which does not increase again until the first embryonic cleavage some 90 min later (20, 21). Mos is also degraded shortly after fertilization, following the disappearance of cyclin and MPF (51). During early cleavages the embryo rapidly cycles between the S and M phases, with MPF activity oscillating in accordance with the M phase. Mos protein is essentially undetectable in cleaving embryos.

In the oocyte, Mos is phosphorylated at several serine residues by protein kinases that are active in immature oocytes (18). Mos isolated from mature oocytes is phosphorylated at serine 3. Catalytically inactive mutant Mos is not phosphorylated at this residue, so it may represent a site of autophosphorylation or phosphorylation by a Mos-activated kinase (18, 32). This phosphorylation is important to stabilize Mos from degradation by a ubiquitin-dependent pathway during progression from meiosis I to meiosis II (32). It is not known whether phosphorylation of serine 3 occurs prior to entry into meiosis I.

The unfertilized egg is arrested in metaphase of meiosis II by an activity referred to as cytostatic factor (CSF) (30). Mos is an active component of CSF, which is believed to directly or indirectly stabilize the active form of MPF, resulting in metaphase arrest. Mos RNA or protein injected into one blastomere of a two-cell embryo mimics CSF and arrests

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cleavage of the injected half of the embryo at the metaphase of mitosis (43, 51). Moreover, the CSF activity in egg extracts is neutralized with Mos antibodies (43). However, the Ras oncoprotein can also arrest embryo cleavage at mitotic metaphase, independently of Mos (8, 9).

A mitogen-activated protein (MAP) kinase, Xp42, is also activated during oocyte maturation (17, 23, 24, 38, 54). This kinase preexists in the oocyte and is activated during maturation by dual phosphorylation on a tyrosine and a threonine residue (37). MAP kinase is activated contemporaneously with or slightly after MPF and lags about 2 h behind the onset of Mos protein synthesis (17, 24, 38, 41). MAP kinase is dephosphorylated approximately 20 min following egg fertilization, at about the time of Mos protein degradation, and MAP kinase is not rephosphorylated during early embryogenesis (17, 38, 54). MPF activity is reactivated in subsequent mitotic cell cycles, although neither Mos nor MAP kinase is reactivated during embryogenesis. Hence, the activities of Mos and MAP kinase appear to be coordinately regulated throughout oocyte maturation and early embryogenesis.

In somatic cells, MAP kinases are activated by various extracellular stimuli (reviewed in references 5, 35, and 49). Activation requires dual phosphorylation on a threonine and a tyrosine residue (34, 37), catalyzed by a specific activator (1, 22, 29, 31, 37). Since no systematic nomenclature exists for these various activators, they have been referred to operationally as MAP kinase kinases (MAPKKs) or MAP kinase activators (reviewed in reference 2). The cDNA for a murine MAPKK, termed MEK, has recently been cloned (7). MEK is most closely related by sequence to the serine/threonine kinases, although it is able to activate MAP kinase by phosphorylating the regulatory threonine and tyrosine residues (7). The MAPKKs are themselves regulated by phosphorylation at serine and threonine residues and are inactivated by treatment with serine/threonine-specific protein phosphatases (22). It appears that the serine/threonine protein kinase Raf is able to phosphorylate and reactivate a phosphatase-inactivated MAPKK (11, 26, 28). Activating this cascade of kinases in tissue culture cells by polypeptide mitogens and hormones requires functional Ras protein (12, 33, 50, 52).

We were interested to determine whether any functional interaction exists between Mos and MAP kinase. In this report we demonstrate that Mos protein induces rapid activation of MAP kinase in oocytes as well as during Mos-induced mitotic arrest in early embryos. This activation appears to act through a physiological MAPKK. Furthermore, Mos is able to directly phosphorylate and activate a purified, phosphatase-inactivated MAPKK.

MATERIALS AND METHODS

Reagents. Wild-type (XE) and kinase-inactive (KM) Mos were fused to MBP. Fusion proteins were expressed in and purified from *Escherichia coli* as described previously (53). Recombinant ERK2 protein was expressed and purified from *E. coli* as described previously (44). RNA encoding epitope-tagged kinase-inactive mutant MAP kinase (mt-Xp42-K57R) was made as described previously (53).

Oocyte microinjections. A *Xenopus* ovary was digested in MBS (88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.8], 2.5 mM $NaHCO_3$) containing 2 mg of collagenase per ml. Stage 6 oocytes were incubated for 60 min in MBS containing cycloheximide (10 μg/ml) prior to injection with approximately 50 nl of MBP-Mos fusion protein at 0.3 mg/ml in 88 mM NaCl–20 mM HEPES [pH 6.8]–10 mM maltose. Following injections, some oocytes were treated with progesterone (10 μg/ml). For assays of MPF and MAP kinase activity, groups of 10 oocytes were homogenized at each time point in CB (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 mM vanadate, 0.5 μM microcystin). For embryo injections, ovulated eggs were fertilized with freshly removed minced testis and briefly dejellied in 2% cysteine (pH 7.5). Dejellied embryos were treated with 10 washes of MBS to ensure that all the cysteine was removed. Embryos were injected 90 min later, at the time of the first cell cleavage, in one cell of the two-cell embryo. Embryos were incubated at 25°C for 30 min before being homogenized in CB. At this time, the control blastomere had divided while the injected one had not. Homogenates were clarified by centrifugation for 20 min at high speed in a microcentrifuge.

Immunoprecipitation of MAP kinase. Extracts of 10 oocytes for each time point were immunoprecipitated with anti-MAP kinase antibody 1913 as described previously (37). For immunoblotting, proteins were transferred to Immobilon (Whatman) polyvinylidene difluoride membrane, blocked with 1% bovine serum albumin, and probed with anti-MAP kinase antibody 1913 at a 1:2,000 dilution.

In vitro kinase assays. To assay MAP kinase activity, we used glutathione-S-transferase (GST)-Myc as an in vitro substrate (37). Assays were done by resuspending immunoprecipitates in 30 μl of CB containing GST-Myc (2 μg) and [γ-^{32}P]ATP (10 μCi). MAPKK assays were done by incubating equal oocyte equivalents of extract (10 oocytes per time point) for 30 min in CB containing [γ-^{32}P]ATP (10 μCi) and recombinant ERK2 protein (0.6 μg). MPF activity was determined by affinity purification of p34-cyclin B complexes with p13^{suc1} Sepharose as described previously (20). The complexes were incubated in CB containing 1 μg of histone H1 (Boehringer) and 10 μCi of [γ-^{32}P]ATP.

In vitro phosphorylation of MAPKK by MBP-Mos. To assay the ability of Mos to activate MAPKK in vitro, purified wild-type and kinase-inactive MBP-Mos fusion proteins (1.2 μg) were incubated for 60 min at 30°C in rabbit reticulocyte lysate (400 μl) containing 7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 1 mM $MgCl_2$, and 2.5 μM microcystin. The reticulocyte lysate was diluted with phosphate-buffered saline–0.1% Triton X-100–80 mM β-glycerophosphate, and activated MBP-Mos was immunoprecipitated by incubation with 10 μl of 5S antibody for 1 h at 0°C. Protein G Sepharose (20 μl) was then added and mixed at 4°C for 30 min, and the mixture was washed three times with the dilution buffer. The 45-kDa form of MAPKK was purified approximately 20,000-fold from rabbit skeletal muscle by the method of Seger et al. (44), through to the MonoS step, with the exception that a batchwise DEAE-cellulose adsorption was included as the first step. MAPKK was inactivated by treatment with 50 U of phosphatase 2A per ml, purified from beef heart as described previously (1), for 30 min at 30°C. The phosphatase was then inhibited with 1 μM microcystin. The kinase reactions (volume, 50 μl) contained wild-type or kinase-inactive MBP-Mos, 10 mM $MgCl_2$, 100 μM ATP, 20 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), 0 or 1 μg of ERK2, and 0 or 15 ng of MAPKK. Phosphorylation was carried out at 25°C for 15 min and then at 30°C for 15 min. The products

were separated on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel and detected by autoradiography.

RESULTS

Mos activates MAP kinase in *Xenopus* oocytes. MBP-Mos fusion proteins were injected into oocytes to determine whether Mos kinase activity had any effect on the *Xenopus* MAP kinase, Xp42. The phosphorylation state of MAP kinase was monitored by its migration on SDS-polyacrylamide gels. Phosphorylation of Xp42 at either T188 or Y190 or both causes a characteristic reduction in its migration through SDS-polyacrylamide gels, resulting in an apparent 2-kDa increase in molecular mass, or band shift. Since phosphorylation at one site will cause the band shift but is not sufficient to increase catalytic activity, in vitro kinase assays were used to assay the catalytic activity of MAP kinase.

Induction of meiotic maturation by MBP-Mos these conditions is enhanced by progesterone and is not inhibited by cycloheximide. Progesterone does not induce maturation when protein synthesis is blocked by cycloheximide, unless MBP-Mos is injected (53). Injection of wild-type Mos fusion protein (MBP-Mos XE) resulted in rapid activation of MAP kinase catalytic activity whereas injection of kinase-inactive Mos (MBP-Mos KM) did not (Fig. 1A). The Mos-induced activation of MAP kinase resulted in phosphorylation of approximately 80% of the MAP kinase molecules 1 h after injection, as evidenced by the band shift on immunoblots of immunoprecipitated MAP kinase (Fig. 1B, panel a, lane 3). For comparison, treatment of oocytes with progesterone, in the absence of cycloheximide, caused phosphorylation of 10 to 20% of MAP kinase after 1 h and 100% after 16 h, at which time GVBD was complete (Fig. 1B, panel a, lanes 2 and 6). The phosphorylated MAP kinase in MBP-Mos-injected oocytes was presumably phosphorylated on both the regulatory threonine and tyrosine residues, since its catalytic activity was comparable to that observed in progesterone-matured oocytes (Fig. 1B, panel b, lanes 3 and 6). In addition, this phosphorylation appeared to be carried out by a *Xenopus* MAPKK, since extracts of oocytes injected with wild-type Mos kinase had a MAPKK activity and were able to phosphorylate recombinant ERK2 (Fig. 1B, panel c, lane 3). In contrast, the extracts of oocytes injected with kinase-inactive Mos were not able to phosphorylate ERK2 (Fig. 1B, panel c, lane 4).

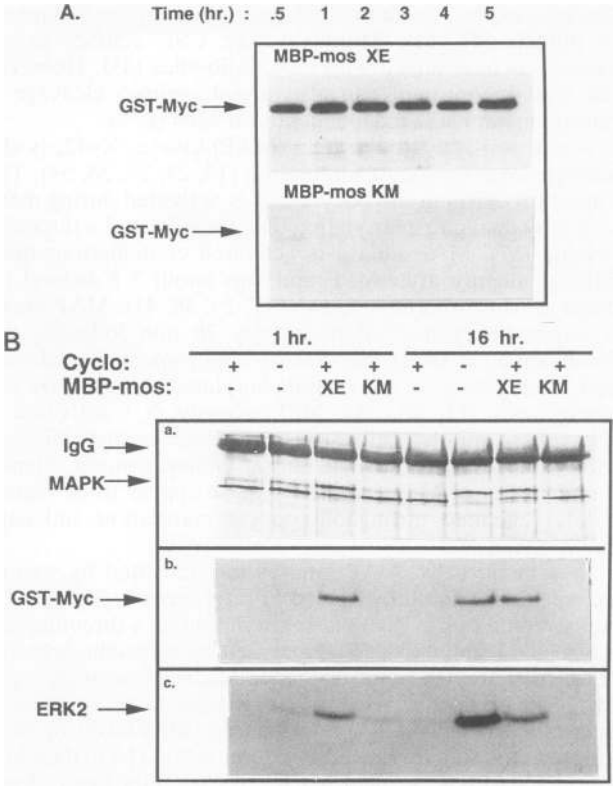

FIG. 1. Injection of MBP-Mos XE activates MAP kinase. (A) Wild-type (XE) or kinase-inactive (KM) Mos fusion protein was microinjected into cycloheximide-pretreated *Xenopus* oocytes, and progesterone was added. At the specified time points, oocytes were extracted, Xp42 was immunoprecipitated, and GST-Myc was used as a substrate for in vitro kinase assays. (B) Wild-type or kinase-inactive Mos fusion proteins were microinjected into control or cycloheximide-pretreated *Xenopus* oocytes. All oocytes were subsequently treated with progesterone. Extracts were prepared at either 1 or 16 h following progesterone treatment, and MAP kinase was immunoprecipitated. Panel a shows an Xp42 immunoblot of Xp42 immunoprecipitates. Panel b shows in vitro kinase assays on the same immunoprecipitates with GST-Myc as a substrate. Panel c shows assays of oocyte extracts for MAP kinase activator activity with recombinant ERK2 as a substrate.

These results suggested that MBP-Mos could rapidly activate MAP kinase and a MAPKK, in the absence of protein synthesis. We next compared the kinetics of activation of MAP kinase and MPF. Oocytes were injected with MBP-Mos in the absence of both cycloheximide and progesterone. Under these conditions, MBP-Mos induces complete maturation (53). Control oocytes were not injected but were incubated with progesterone. At various times, 10 oocytes were homogenized and assayed for $p34^{cdc2}$-associated kinase activity, following affinity purification with $p13^{suc1}$-agarose, and for MAP kinase activity, following immunoprecipitation with antibody to Xp42. Progesterone resulted in 100% GVBD within 6.5 h and 50% GVBD by 4.5 h (Fig. 2A). Injection of wild-type MBP-Mos (XE) induced 100% GVBD within 4.5 h and 50% GVBD by 3.5 h. Injection of wild-type MBP-Mos fusion protein induced phosphorylation of about 50% of the Xp42 (Fig. 2B), and stimulated MAP kinase catalytic activity (Fig. 2C), within 20 min following injection. Phosphorylation of Xp42 was complete by 2 h, and catalytic activity remained high throughout the time course. In contrast, MPF, measured by histone H1 kinase activity, was not elevated until approximately 2 h following injection of the wild-type MBP-Mos fusion protein (Fig. 2D). Kinase-inactive MBP-Mos (KM) was not able to induce GVBD, MAP kinase phosphorylation, MAP kinase activation, or MPF activity.

To determine whether MAP kinase was being phosphorylated via the physiological phosphorylation cascade, involving a MAPKK, oocytes were given an injection of RNA encoding an epitope-tagged version of kinase-inactive Xp42 (mt-Xp42-K57R) and then, 36 h later, an injection of MBP-Mos fusion proteins. Extracts were prepared 1 h later. The level of ectopic expression of mt-Xp42-K57R appeared to be approximately equal to the endogenous level of Xp42 (Fig. 3A, lanes 3 and 4). In addition, mt-Xp42-K57R was stoichiometrically phosphorylated and band shifted in response to microinjection of wild-type but not kinase-inactive MBP-Mos (Fig. 3A, lanes 3 and 4). These results suggest that MAP kinase was being activated by another kinase, presumably a MAPKK. Moreover, expressing mt-Xp42-K57R did not in-

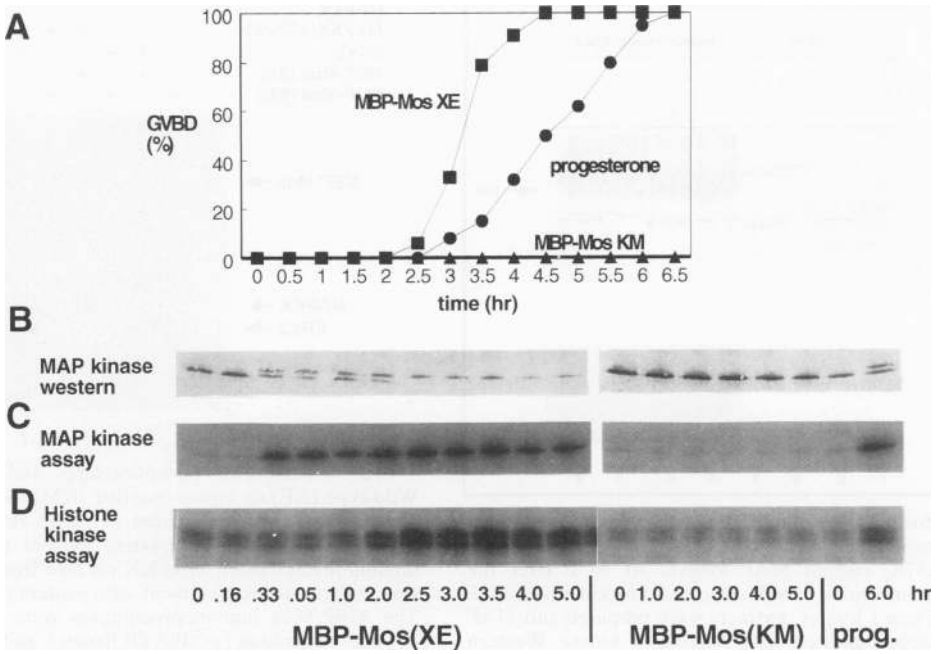

FIG. 2. Kinetics of activation of MAP kinase and MPF. Oocytes were given injections of wild-type (XE) or kinase-inactive (KM) MBP-Mos fusion proteins or were incubated with progesterone. Groups of 10 oocytes were homogenized at each time point. MAP kinase was immunoprecipitated, and in vitro kinase assays were done on the immunoprecipitates with GST-Myc as a substrate. To measure MPF activity, $p34^{cdc2}$ was affinity purified with $p13^{suc1}$-agarose and in vitro kinase assays were performed with histone H1 as a substrate. (A) Percentage of oocytes showing GVBD. (B) Immunoblot of immunoprecipitated Xp42 with antibody to Xp42. (C) Phosphorylation of GST-Myc by immunoprecipitated Xp42. (D) Histone H1 phosphorylation by $p13^{suc1}$-associated kinases.

terfere with phosphorylation of the endogenous Xp42, and both proteins were quantitatively phosphorylated and band shifted (Fig. 3A, lanes 3 and 4). These results are similar to the effects of activating the usual phosphorylation cascade with progesterone (37), suggesting that a physiological MAPKK is being activated by Mos.

Mos is able to activate a purified MAPKK in vitro. We tested the ability of Mos to phosphorylate and activate a MAPKK in vitro. Wild-type and kinase-inactive MBP-Mos were treated by incubation in rabbit reticulocyte lysate and purified by immunoprecipitation. Activity was evidenced by autophosphorylation of the wild-type but not the kinase-inactive MBP-Mos (Fig. 4, lanes 1 and 2). As a substrate, we purified MAPKK from rabbit skeletal muscle. MAPKK was able to phosphorylate a MAP kinase, ERK2 (Fig. 4, lane 10; note that the MAPKK is also a substrate for ERK2 [compare lanes 9 and 10]). The phosphorylation of ERK2 by MAPKK was greatly decreased by prior incubation of MAPKK with protein phosphatase 2A, followed by the phosphatase inhibitor microcystin (lane 12). After incubation of phosphatase-treated MAPKK with wild-type but not kinase-inactive MBP-Mos, phosphorylation of MAPKK was detected (lanes 5 and 6). This phosphorylation was presumably on the regulatory residue(s) since it activated the phosphorylation of ERK2 (lanes 7 and 8). In the absence of MAPKK, wild-type MBP-Mos phosphorylated ERK2 only weakly (lane 3), and only trace ERK2 autophosphorylation was evident with kinase-inactive MBP-Mos (lane 4). The phosphorylation of MAPKK by MBP-Mos apparently proceeded to high stoichiometry, since it phosphorylated the ERK2 substrate nearly as well as fully active MAPKK (compare lanes 7 and 10). We do not know the efficiency of the reaction, however, since the rates of phosphorylation of MAPKK by MBP-Mos were not measured.

Mos-induced mitotic arrest results in MAP kinase phosphorylation. It is known that Mos is the active component of CSF, and injecting Mos into one blastomere of a two-cell embryo has the dramatic effect of arresting mitotic divisions in that blastomere (43, 53). We were interested in learning whether the signaling pathway activated by Mos and present in the unfertilized egg was intact following fertilization. Therefore, we injected MBP-Mos fusion proteins into one blastomere of a two-cell embryo. By 30 min later arrest of the injected blastomeres was evident, and we immunoprecipitated Xp42 from the embryos. Arresting mitosis with wild-type Mos resulted in Xp42 phosphorylation and activation of catalytic activity (Fig. 5, lane 3). Injecting kinase-inactive Mos did not activate MAP kinase (lane 2).

DISCUSSION

Injecting soluble MBP-Mos fusion proteins into *Xenopus* oocytes activates both a MAPKK and the MAP kinase, Xp42, prior to and apparently independent of MPF activation. These effects of Mos require a functional kinase domain and presumably occur as a result of the phosphorylation of one or more oocyte proteins by the injected MBP-Mos. Furthermore, the MBP-Mos fusion protein induces the in vitro phosphorylation of a MAPKK protein, extensively purified from rabbit muscle and inactivated with phosphatase. This phosphorylation reactivates the MAPKK, as detected by increased phosphorylation of MAP kinase. The phosphorylation of MAPKK by Mos proceeds to high stoichiometry, since most of the original activity is restored. Thus, Mos may be a "MAP kinase kinase kinase" and may normally activate MAP kinase during oocyte maturation and entry into meiosis. Moreover, we find that MBP-Mos activates MAP kinase in *Xenopus* cleavage embryos concomi-

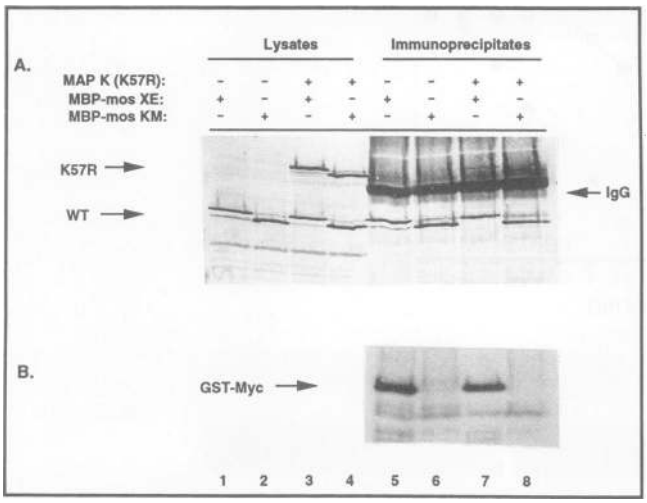

FIG. 3. MBP-Mos activates the MAP kinase activator. *Xenopus* oocytes were microinjected with RNA encoding epitope-tagged kinase-inactive (K57R) mutant MAP kinase. At 36 h later the oocytes were given injections of wild-type (XE) or kinase-inactive (KM) MBP-Mos. Then 1 h later, extracts were prepared and MAP kinase was immunoprecipitated. (A) Anti-MAP kinase Western immunoblot of lysates and anti-MAP kinase immunoprecipitates. The upper arrow points to the ectopically synthesized kinase-inactive MAP kinase, and the bottom arrow points to the endogenous wild-type MAP kinase. IgG, immunoglobulin G. (B) In vitro kinase assays on immunoprecipitates 5 to 8 from panel A. GST-Myc was used as a substrate.

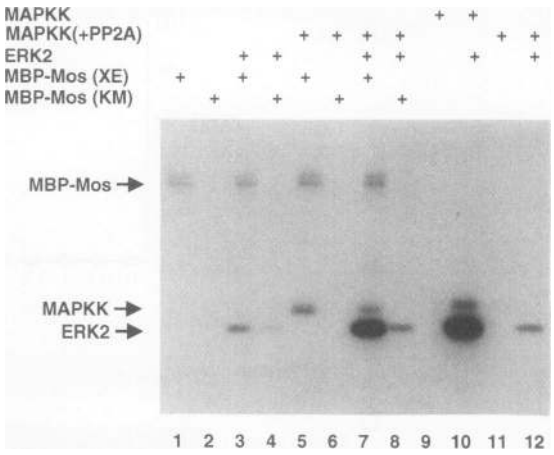

FIG. 4. MBP-Mos phosphorylates and activates a MAPKK. Wild-type (XE) or kinase-inactive (KM) MBP-Mos fusion proteins were activated by incubation in rabbit reticulocyte lysate in the presence of an ATP-generating system and then repurified by immunoprecipitation. MAPKK purified from rabbit skeletal muscle was inactivated by treatment with protein phosphatase 2A (PP2A). The MBP-Mos immunoprecipitates were incubated in a kinase cocktail containing [γ-^{32}P]ATP (lanes 1 and 2); the arrow indicates autophosphorylation of the MBP-Mos XE. The immunoprecipitates were mixed with recombinant ERK2 (lanes 3 and 4); the arrow indicates a small amount of phosphorylation of ERK2 by MBP-Mos XE and a lesser amount of autophosphorylation of ERK2 in the presence of MBP-Mos-KM. In lanes 5 and 6 the Mos immunoprecipitates were mixed with protein phosphatase 2A-inactivated MAPKK; the arrow indicates phosphorylation of the MAPKK. In lanes 7 and 8 the immunoprecipitates were mixed with inactivated MAPKK and ERK2; the arrow indicates phosphorylation of the MAPKK as well as ERK2. In lanes 9 to 12, the MBP-Mos immunoprecipitates were omitted and untreated (lanes 9 and 10) or PP2A-treated (lanes 11 and 12) MAPKK was incubated in the absence (lanes 9 and 11) or presence (lanes 10 and 12) of ERK2.

tant with arrest at mitotic metaphase. MAP kinase activation has not been detected during normal mitosis, but it does accompany meiotic metaphase (6, 17). Since Mos is normally present at meiosis but not mitosis, whereas MPF is present in both situations, we suggest that Mos, not MPF, may govern MAP kinase activity at meiotic metaphase.

Previous results have implicated MPF in the control of MAP kinase at meiosis. In progesterone-treated oocytes, MAP kinase and MPF activities increase in concert at the time of entry into metaphase (17, 24), about 2 h after Mos protein synthesis is detected (41). If newly synthesized Mos is enzymatically active, and if Mos activates MAP kinase, MAP kinase activation should be detected earlier. One possibility is that newly synthesized Mos is inactive until cyclic AMP (cAMP) levels are reduced, since the cAMP-dependent protein kinase has been identified as a downstream antagonist of Mos (10). Endogenous Mos may also be localized, so as to restrict its access to MAPKK. Mos is complexed with tubulin in a high-molecular-weight complex (56), which could localize Mos within the oocyte and delay the activation of MAP kinase.

Evidence that MPF can activate MAP kinase comes from the observation that injection of crude or partly purified MPF into *Xenopus* oocytes, or addition of purified MPF to interphase extracts, activates MAP kinase, MAPKK, and endogenous pre-MPF in the absence of protein synthesis (17, 23, 29). It is possible, however, that these preparations of MPF, derived from unfertilized eggs, were contaminated with Mos, because Mos physically associates with a cyclin-dependent kinase in Mos-transformed tissue culture cells (55). However, MAP kinase is also activated in interphase extracts, which lack Mos, when MPF is created by the addition of bacterially synthesized cyclin (45). Therefore, MAP kinase can be activated by a Mos-independent, MPF-dependent mechanism or by a Mos-dependent, MPF-independent pathway (described here), and the importance of each may depend on the conditions.

One pathway for MAP kinase activation in tissue culture cells has been discovered recently. Both MAP kinase and

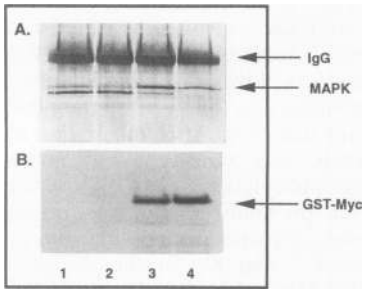

FIG. 5. Mos-induced MAP kinase activation during mitotic arrest of embryos. Two-cell embryos were either untreated (lane 1) or given injections of kinase-inactive (lane 2) or wild type (lane 3) MB-Mos and incubated for 30 min. Extracts were prepared, and MAP kinase immunoprecipitated. As a positive control, a similar amount of egg extract was made and immunoprecipitated (lane 4). (A) Immunoblot of Xp42 immunoprecipitates probed with antibody to Xp42. IgG, immunoglobulin G. (B) In vitro kinase assays on the same immunoprecipitates with GST-Myc as a substrate.

MAPKK are activated by oncogenic mutant Ras, and their activation by nerve growth factor, insulin, or platelet-derived growth factor requires the endogenous Ras protein (12, 33, 39, 50, 52). In mammalian cells, Ras seems to act on the Raf protein kinase, directly or indirectly, because (i) activation of MAP kinase by Raf is independent of Ras, (ii) Raf is activated in Ras-transformed cells, (iii) inhibition of Raf blocks MAP kinase activation by Ras or growth factors, and (iv) activated mutant Raf activates MAP kinase (11, 26, 28). In vitro, immunopurified or recombinant Raf can phosphorylate and reactivate phosphatase-inactivated MAPKK (11, 26, 28). Therefore, Raf may be a MAP kinase kinase kinase, although the possibility of contamination of one of the reagents with yet another kinase is difficult to rule out.

The Ras pathway of MAP kinase activation may also operate in oocytes. *Xenopus* oocytes contain Ras, which is required for insulin-stimulated maturation (27). Furthermore, oncogenic mutant Ras induces maturation in the absence of hormonal stimulation (4). However, endogenous Ras appears not to be required for progesterone-induced maturation (13), and although Mos is required for GVBD induced by progesterone, it is not required for GVBD induced by the Ras oncoprotein (8). Thus, there appear to be Ras-dependent and -independent pathways to maturation. The Ras oncoprotein also stimulates MAP kinase and MAPKK in intact oocytes (25). This probably accounts for the observation that S6 kinase II is activated in oocytes injected with Ras oncoprotein (3), because S6 kinase II is activated in vitro by MAP kinase (48; reviewed in reference 47). In oocyte extracts, the Ras oncoprotein stimulates MAP kinase independently of Mos and MPF activation (25, 45). However, in oocytes injected with Ras oncoprotein, S6 kinase II activation is inhibited partly by Mos antisense oligonucleotides, suggesting that both Mos-dependent and -independent pathways of MAP kinase activation by Ras exist (3). We have found that the injection of recombinant dominant-negative mutant Ras (Asn-17) into oocytes did not block the MBP-Mos-induced MAP kinase activation (36), suggesting that Mos does not work through Ras to induce MAP kinase activation. Thus, in addition to Mos- and MPF-dependent mechanisms, there may be a third, Ras-dependent pathway for MAP kinase activation. Whether Raf is involved in some or all of these pathways awaits further experiments.

Our in vitro studies imply that MBP-Mos is a direct activator of MAPKK, like Raf. There are other possibilities, however. MBP-Mos appears to be inactive, when purified from bacteria, in both autophosphorylation and phosphorylation of MAPKK (reference 53 and data not shown). After incubation in extracts of oocytes or reticulocytes supplemented with an ATP-generating system, MBP-Mos becomes capable of autophosphorylation (53) or of phosphorylating MAPKK. This requirement for "activation" of MBP-Mos may explain the 10- to 20-min delay in MAP kinase phosphorylation when MBP-Mos was injected into oocytes. The need for activation of MBP-Mos in reticulocyte lysate means that one or more reticulocyte-derived protein kinases may be responsible for the phosphorylation of MAPKK detected in vitro. This seems unlikely, because the MBP-Mos was purified by immunoprecipitation and washing with detergents. If the MBP-Mos is contaminated with another protein kinase, the phosphorylation of MAPKK in vitro may not be catalyzed by Mos directly but by a Mos-associated kinase. The absence of phosphorylation by kinase-inactive mutant MBP-Mos requires that any such Mos-associated kinase be regulated by Mos.

Injecting Mos RNA or MBP-Mos protein into oocytes is sufficient to drive the oocytes through meiosis into metaphase arrest. Similarly, injecting Mos RNA or protein into one cell of a two-cell blastula arrests the injected half of the embryo in mitotic metaphase, while the uninjected half continues dividing. In vitro, Mos can phosphorylate the cyclin B2 component of MPF (40), but the biochemical basis of the cytostatic effect is unknown. Our data suggest that MAP kinase may be a mediator of the cytostatic effect of Mos. Shortly after injection of MBP-Mos into either oocytes or embryos, MAP kinase becomes phosphorylated and catalytically active. Although purified, active MAP kinase does not activate pre-MPF in oocyte extracts (23), MAP kinase may cooperate with MPF and Mos to contribute to metaphase arrest. It is interesting that the Ras oncoprotein also induces metaphase arrest (8, 9) and MAP kinase activation (25, 45). Thus, the induction of oocyte maturation and metaphase arrest by Ras and Mos may converge at the level of MAP kinase activation. In vitro, MAP kinase phosphorylates a microtubule-associated protein and causes interphase microtubules to resemble metaphase microtubules (24, 46), and it has been proposed that Mos kinase is involved in the reorganization of microtubules that leads to spindle assembly (56, 57). It is possible that Mos, Ras, and MPF, by activating MAP kinase, promote formation of the spindle.

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ADDENDUM IN PROOF

A. Nebreda and T. Hunt have found that a similar MBP-Mos protein activates MAP kinase in extracts of *Xenopus* oocytes (EMBO J., in press).

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