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The protein kinase *mos* activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo

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The *mos* protooncogene encodes a serine/threonine protein kinase that is only expressed at significant levels in germ cells. Recombinant *malE-mos* protein (*Xenopus mos* protooncogene fused in frame to the maltose binding protein of *E. coli*) activates MAP kinase in cell-free extracts prepared from *Xenopus* oocytes and eggs. Here we show that *malE-mos* immunoprecipitates from *Xenopus* extracts phosphorylate and activate MAP kinase kinase in vitro, indicating that *mos* can function as a MAP kinase kinase kinase. Moreover, ectopic expression of *mos* in mammalian somatic cells, that lack any endogenous *mos* protein, triggers the activation of MAP kinase in vivo. These results identify the *mos* protooncogene as a direct activator of the MAP kinase pathway, with the potential to activate this kinase cascade even in cells where normally there is no expression of *mos*.

MAP kinase; *mos*; Elk-1; Oncogene; Protein phosphorylation; Signal transduction

1. INTRODUCTION

Mitogen activated protein (MAP) kinases comprise a family of serine/threonine protein kinases conserved through evolution in all eukaryotic cells from yeast to humans, that participate in signal transduction pathways initiated by many extracellular stimuli [1–4]. The activation of MAP kinases requires concomitant phosphorylation on specific threonine and tyrosine residues [5,6], catalysed by a dual specificity MAP kinase kinase [7–9]. MAP kinase kinase is in turn activated by serine/threonine phosphorylation [10], catalysed by MAP kinase kinase kinases [11–15]. While the MAP kinase kinases identified so far are quite similar in structure and properties [16], there is more divergence at the level of MAP kinase kinase kinases, suggesting that the protein kinase cascade may integrate several different inputs at this point [15].

MAP kinase is activated during the meiotic maturation of *Xenopus* oocytes induced by progesterone [17–19]. We and others recently found that the *mos* protooncogene, a serine/threonine protein kinase that is only expressed at high levels in germ cells and whose synthesis is required for progesterone-induced *Xenopus* oocyte maturation [20], can activate MAP kinase in *Xenopus* cell-free extracts [21] and intact oocytes [22]. Here we report that *mos* immunoprecipitates from *Xenopus* cell-free extracts can directly phosphorylate and activate MAP kinase kinase in vitro. In addition we show that expression of *Xenopus mos* in mammalian somatic cells (NIH 3T3 fibroblasts) can activate the MAP kinase pathway.

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2. MATERIALS AND METHODS

2.1. *Preparation of active* malE-mos *protein*

Recombinant *malE-mos* fusion protein was purified from *E. coli* as described [21], and was activated by incubation in interphase *Xenopus* cell-free extracts. Interphase extracts were prepared by treating CSF-arrested egg extracts [23] with 0.4 mM $CaCl_2$ for 60 min at 24°C, followed by 0.4 mM EGTA for 5 min. Typically, 1 μg of purified *malE-mos* was incubated in 20 μl of interphase extract for 50–60 min at 24°C. The extract was diluted 10-fold with ice-cold H1 kinase buffer (80 mM β-glycerophosphate, 15 mM $MgCl_2$, 20 mM EGTA) and activated *malE-mos* was immunoprecipitated by incubation with 3 μl of anti-*malE* antibody (New England Biolabs) for 3 h on ice. The immunoprecipitates were recovered by incubation with protein A for 30 min at 4°C, and then washed 3 times with a buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween and protease inhibitors (1 mM PMSF, 2 mM benzamidine, 10 μg/ml leupeptin and 10 μg/ml aprotinin).

2.2. *Phosphorylation of MAP kinase kinase by* mos *in vitro*

Purified MAP kinase kinase from rabbit skeletal muscle [24] was inactivated by treatment with 30 mU of protein phosphatase 2A per ml for 30 min at 30°C. The phosphatase was then inhibited with 3 μM okadaic acid. For the kinase reaction, *malE-mos* immunoprecipitates were washed once in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.04% Brij-35, and then incubated for 30 min at 30°C with 150 ng of the inactivated MAP kinase kinase, 10 mM magnesium acetate and 200 μM [γ-^{32}P]ATP (10^6 cpm/nmol) in a total volume of 20 μl. Phosphorylations were analysed by SDS-PAGE and autoradiography.

2.3. *Activation of MAP kinase kinase by* mos *in vitro*

MalE-mos immunoprecipitates were washed once in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.04% Brij-35, and then incubated in a final volume of 40 μl with 200 μM ATP, 10

mM magnesium acetate, 1 μM inactive 42 kDa isoform of MAP kinase ($p42^{mapk}$) expressed in *E. coli* [25], and either with or without inactive MAP kinase kinase isolated by MonoQ chromatography from unstimulated PC-12 cells [14]. In some experiments we used inactive MAP kinase kinase expressed in *E. coli* (S. Traverse et al., submitted). After 30 min at 30°C, 10 μl of the reaction supernatant, was diluted 5-fold into icc-cold 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.5 mM Na_3VO_4, 1 mg/ml BSA, and assayed for MAP kinase activity with myelin basic protein (MBP) substrate as described previously [10].

2.4. *Phosphorylation and transcriptional activation of Elk-1 in mouse fibroblasts transfected with* mos

To express *mos* in mammalian somatic cells, a 1.2 kb *Eco*RI-*Xho*I DNA fragment containing the full-length *Xenopus mos* protooncogene, was inserted between the *Eco*RI and *Xho*I sites of the MLVβplink expression vector [26]. The resulting plasmid MLV-Mos was transfected into NIH 3T3 mouse fibroblasts by the DEAE-dextran protocol as described previously [27]. To study the phosphorylation of the Elk-1 transcription factor, fibroblasts were cotransfected with MLV-Mos, MLV-Ras^{Val12} or a control plasmid MLVβ128 [27] and the plasmid NL.Elk, which encodes a fusion protein in which the LexA DNA binding domain replaces the Ets domain in Elk-1 [27]. To study the effect of *mos* on Elk-1 transcriptional activity, fibroblasts were cotransfected with MLV-Mos, MLVNlex.ElkC, in which the C-terminal region of Elk-1 is fused to the entire LexA coding region, and the reporter plasmid $(LEX\ OP)_2TK{\cdot}CAT$ [28]. Following transfection, cells were serum deprived for 40 h, and then extracts were prepared and either analysed by western blotting with anti-LexA antiserum or assayed for CAT activity [27,28].

2.5. *Activation of MAP kinase in mouse fibroblasts expressing* mos

For preparation of a cell line expressing the *Xenopus mos* protooncogene, NIH 3T3 cells were cotransfected with 10 μg of MLV-Mos and 1 μg of pMEXneo [29], using the calcium phosphate precipitation technique. As a control, another cell line was prepared by transfection with the vector MLV-βplink and pMEXneo. Colonies of G418-resistant cells were isolated, grown in 10% serum and finally serum deprived for 24 h before analysis. Cell extracts were prepared in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 20 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 1% NP-40 and the same protease inhibitors as described above. The phosphorylation state of $p42^{mapk}$ and $p44^{mapk}$ was determined by immunoblotting with a commercial monoclonal antibody, as recommended by the supplier (Zymed). MBP kinase activity was assayed as described [21,30], using either total extracts or $p42^{mapk}$ immunoprecipitates prepared with the anti-$p42^{mapk}$ antiserum 122 [31].

3. RESULTS AND DISCUSSION

We have previously demonstrated that activation of MAP kinase by *mos* in *Xenopus* cell-free extracts involves MAP kinase kinase activation [21]. We were therefore interested in determining whether *mos* could directly activate MAP kinase kinase. Recombinant *malE-mos* protein is inactive as purified from bacteria but can be activated (by an unknown mechanism) by incubation in cell-free extracts prepared from *Xenopus* oocytes and eggs. For the activation experiments, we used interphase extracts to avoid the possibility of contamination with MPF (cyclin B/$p34^{cdc2}$) during the immunoprecipitation. Active *malE-mos* recovered from the extracts by immunoprecipitation with anti-*malE* antibodies showed autophosphorylation activity (Fig. 1, lane 3). The *mos* immunoprecipitates were able to phosphorylate purified MAP kinase kinase that had been dephosphorylated by PP2A treatment (Fig. 1, lane 2). To determine whether the phosphorylation of MAP kinase kinase by *mos* was accompanied by activation, we incubated the *mos* immunoprecipitates with inactive MAP kinase kinase (from unstimulated PC-12 cells) and recombinant $p42^{mapk}$, and then assayed the supernatant for MAP kinase activity. As a control, we prepared immunoprecipitates with the anti-*malE* antibody from untreated *Xenopus* extracts, or with a control antibody from the same *mos*-treated extracts. In such experiments, we typically observed an increase of 8–10-fold in the MAP kinase activity of samples treated with *mos* immunoprecipitates compared with the controls (Fig. 2, compare lane 2 with lane 4). We obtained exactly the same results when the inactive MAP kinase kinase from mammalian cells was replaced in these assays by recombinant MAP kinase kinase purified from bacteria (not shown). In the absence of any MAP kinase kinase, however, the *mos* immunoprecipitates were unable to stimulate the MBP kinase activity of recombinant $p42^{mapk}$ (Fig. 2, lane 3), indicating that *mos* cannot act directly as a MAP kinase kinase. These results also indicate that the *mos* immunoprecipitates were not contaminated with detectable levels of active MAP kinase kinase from the *Xenopus* extracts. Finally, as expected for a process of activation that involves phosphorylation, EDTA abolished the activation of MAP kinase kinase by *mos* (Fig. 2, lane 5).

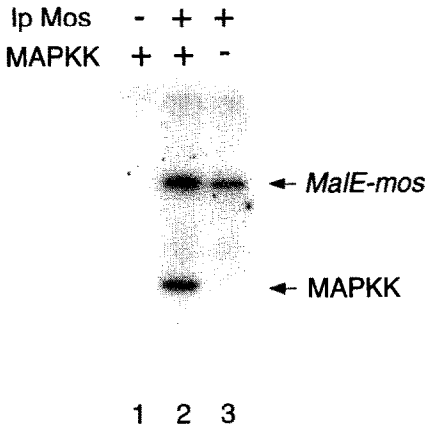

Fig. 1. Phosphorylation of MAP kinase kinase by *mos* in vitro. Purified inactive MAP kinase kinase (lane 1), *malE-mos* immunoprecipitates (lane 3) or both together (lane 2) were incubated in the presence of Mg/[γ-^{32}P]ATP and then analysed by SDS-PAGE and autoradiography.

These results show that *mos* immunoprecipitates can phosphorylate and activate MAP kinase kinase, suggesting that *mos* may work as a MAP kinase kinase kinase. This is probably the way recombinant *mos* triggers the activation of MAP kinase in *Xenopus* cell-free extracts [21]. We cannot rule out the possibility that another protein kinase co-immunoprecipitating with *mos* is actually responsible for the direct phosphoryla-

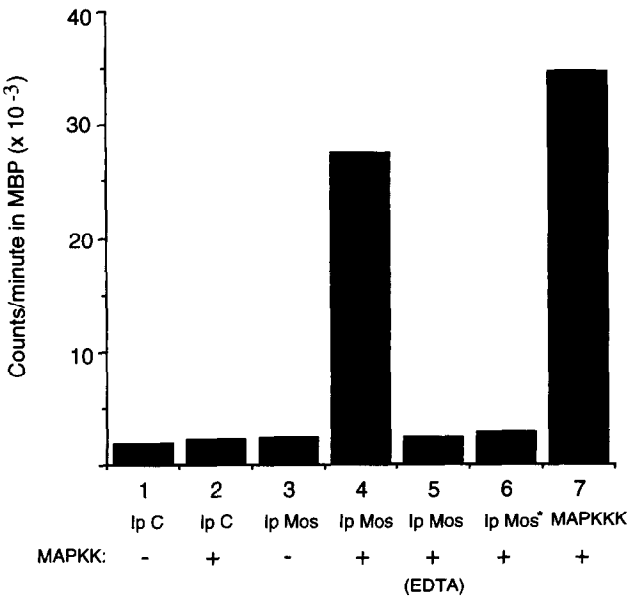

Fig. 2. Activation of MAP kinase kinase by *mos* in vitro. Recombinant p42^{mapk} was incubated with *malE-mos* immunoprecipitates (Ip Mos) or immunoprecipitates prepared with a control antibody (Ip C) in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of inactive MAP kinase kinase, and then assayed for its activity on MBP. As a control, EDTA was added during the incubation of *malE-mos* immunoprecipitates with MAP kinase kinase (lane 5) or during the activation of *malE-mos* (prior to the immunoprecipitation) in the *Xenopus* cell-free extract (lane 6, Ip Mos*). Lane 7, activation with MAP kinase kinase isolated from PC-12 cells [14]. Each lane shows the average of two determinations (standard deviation < 5%). Similar results were obtained in three experiments.

tion and activation of MAP kinase kinase. However, if this is the case, this protein kinase must be specifically associated with and activated by *mos*. In this regard, it is intriguing that *mos* has recently been reported to be required for the activation of Raf-1 (a MAP kinase kinase kinase [11–13]) during *Xenopus* oocyte maturation [32,33]. However, the effect of a dominant negative mutant of Raf-1 on *mos*-induced oocyte maturation [32], could be explained if *mos* and Raf-1 were independently activated by a common mechanism. *Mos* (and other agents that activate the MAP kinase pathway) induce a hyperphosphorylation of Raf-1, but whether this phosphorylation is required for activation of Raf-1 or occurs subsequent to activation is unclear.

The mechanism by which the *mos* protein kinase itself is activated is unknown. When recombinant *malE-mos* is incubated in *Xenopus* extracts supplemented with EDTA, the corresponding *mos* immunoprecipitates can no longer activate MAP kinase kinase in vitro (Fig. 2, lane 6), and also lose their autophosphorylation activity (not shown). These results suggest that *mos* may be activated by phosphorylation. It is generally agreed that *mos* is phosphorylated on serine residues during *Xenopus* oocyte maturation, but there are conflicting reports regarding the importance of phosphorylation for *mos* activity [34,35]. Moreover, there are indications that some MAP kinase kinase kinases might be activated by non-covalent modifications [14]. We have found it difficult to analyse this possibility, because the activity of *mos* immunoprecipitates prepared from *Xenopus* extracts was very labile, completely losing activity, for instance, after 6 min at 30°C. Inactivation was not prevented by addition of 1 μM okadaic acid, suggesting that the lost of activity was not due to dephosphorylation. Interestingly, the *mos* immunoprecipitates could be re-activated by incubation again in the *Xenopus* cell-free extracts, ruling out proteolysis of *mos* as an explanation for the instability. The activation of bacterially produced *malE-mos* was not restricted to *Xenopus* cell-free extracts. We have observed activation of *malE-mos* in extracts prepared from PC-12 cells (not shown) and another group has recently shown activation of a similar *malE-mos* fusion protein upon incubation in rabbit reticulocyte lysates [22]. In agreement with our results, the *mos* immunoprecipitates prepared from reticulocyte lysates could activate MAP kinase kinase in vitro [22].

Since the *mos* protein kinase is normally only expressed at significant levels in germ cells [20], we thought it would be of interest to determine whether ectopic expression of *mos* in somatic cells could also activate the MAP kinase pathway in vivo. For this purpose, we used a novel assay based on the transcription factor Elk-1, which is rapidly phosphorylated at multiple sites in its C-terminal region in response to activation of the MAP kinase pathway in fibroblasts [28]. Elk-1 has been shown to be a substrate for MAP kinase in vitro, and is phosphorylated by a 42 kDa kinase present in extracts from stimulated cells, which may be p42^{mapk} [28]. Cells were cotransfected with a plasmid expressing *Xenopus mos* and a LexA/Elk-1 fusion protein. As shown in Fig. 3, expression of *mos* in NIH 3T3 mouse fibroblasts induced the typical retardation in the

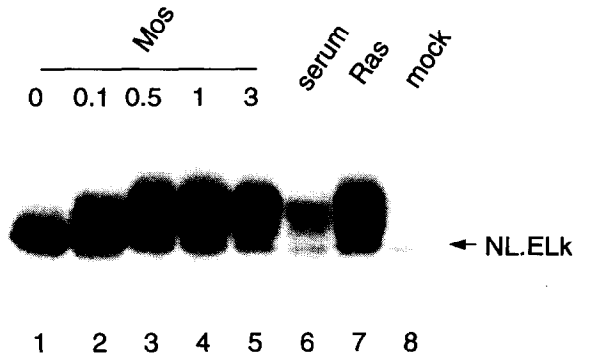

Fig. 3. Phosphorylation of Elk-1 in NIH 3T3 fibroblasts transfected with *mos*. Cell extracts were prepared from fibroblasts transfected with 3 μg of NL.Elk alone (lane 1) or cotransfected with the indicated amounts (μg) of MLV-Mos (lanes 2–5), and analysed by western blotting with anti-LexA antiserum. Lanes 6 and 7, extracts prepared from NL.Elk-transfected fibroblasts stimulated for 15 min with 15% serum and from fibroblasts cotransfected with NL.Elk and MLV-Ras^{Val12}, respectively. Lane 8, extracts prepared from mock-transfected fibroblasts.

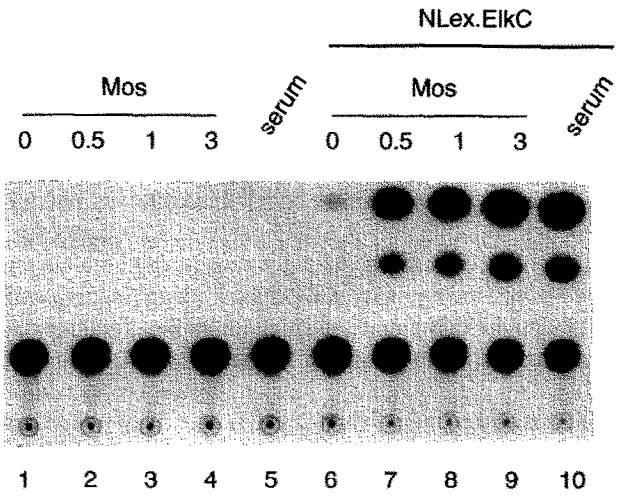

Fig. 4. Activation of Elk-1 in NIH 3T3 fibroblasts transfected with *mos*. Cells were cotransfected with the indicated amounts (μg) of MLV-Mos and 4 μg of the reporter plasmid $(LEX\ OP)_2TK.CAT$ alone (lanes 1–5) or together with 0.25 μg of plasmid NLex.ElkC (lanes 6–10), and then CAT activity was measured. Lanes 5 and 10, fibroblasts were stimulated for 8 h with 15% serum.

electrophoretic mobility of LexA/Elk-1 that accompanies the hyperphosphorylation of the Elk-1 C-terminal region [27]. The activity of *mos* in inducing Elk-1 phosphorylation is similar to that resulting from a 15 min serum stimulation (Fig. 3, lane 6) or constitutive expression of activated *ras*, a well-known activator of the MAP kinase pathway in mammalian fibroblasts [31] (Fig, 3, lane 7). Phosphorylation of the Elk-1 C-terminal region at the MAP kinase sites in response to serum induction of fibroblasts has previously been shown to stimulate its ability to activate transcription. Fig. 4 shows that *mos*-induced phosphorylation of Elk-1 in serum deprived cells stimulated its transcriptional activity to a similar degree.

The phosphorylation and activation of Elk-1 strongly suggests that *mos* is activating the MAP kinase pathway in fibroblasts. To obtain direct evidence for the activation of MAP kinase, we prepared a fibroblast-derived cell line expressing the *Xenopus mos* protooncogene. In this cell line and in the absence of serum stimulation, a significant fraction of both $p42^{mapk}$ and $p44^{mapk}$ show the lower electrophoretic mobility of the activated phosphorylated forms (Fig. 5A). To determine whether the phosphorylation of $p42^{mapk}$ and $p44^{mapk}$ correlated with their activation, we assayed the ability of these extracts to phosphorylate MBP, an in vitro substrate of MAP kinases [30]. As shown in Fig. 5B, the total MBP kinase activity was 2-fold higher in extracts of *mos*-expressing cells than in control cells. This activation was more clearly apparent when we assayed the MBP kinase activity specifically associated with $p42^{mapk}$ immunoprecipitates, which showed a 4–5 fold increase (Fig. 5B).

These results demonstrate that *mos* can stimulate the MAP kinase pathway in mouse fibroblasts, supporting our previous speculation [21] that activation of MAP kinase may be relevant not only for the function of the *mos* protooncogene in germ cells (such as *Xenopus* oocytes) but also for its transforming activity in somatic cells. Although the precise contribution of MAP kinase activation to the process of cellular transformation by oncogenes remains unclear [36], it is difficult to believe that inappropriate activation of such an important and highly conserved element of signal transduction pathways is not connected to the transformation process.

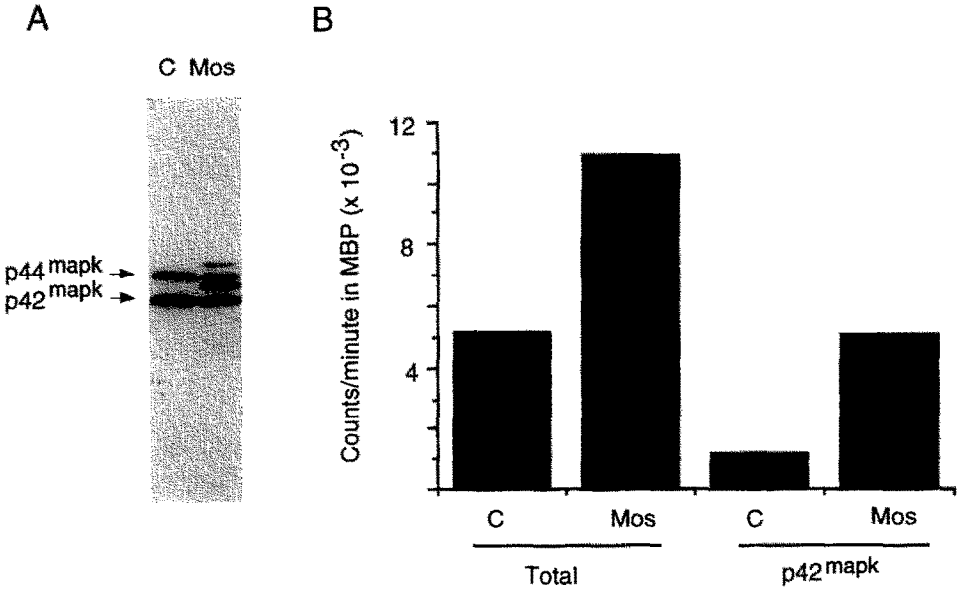

Fig. 5. Activation of MAP kinase in NIH 3T3 fibroblasts expressing *mos*. Cell extracts were prepared from a fibroblast-derived cell line expressing *Xenopus mos* (Mos) or a control cell line (C). (A) Western blotting with the monoclonal anti-MAP kinase antibody. (B) MBP kinase activity in total extracts and in $p42^{mapk}$ immunoprecipitates. Each lane shows the average of two determinations (standard deviation < 3%). Similar results were obtained in two experiments.

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