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Xenopus MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade

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MAP kinase is thought to play a pivotal role not only in the growth factor-stimulated signalling pathway but also in the M phase phosphorylation cascade downstream of MPF. MAP kinase is fully active only when both tyrosine and threonine/serine residues are phosphorylated. We have now identified and purified a *Xenopus* MAP kinase activator from mature oocytes that is able to induce activation and phosphorylation on tyrosine and threonine/serine residues of an inactive form of *Xenopus* MAP kinase. The *Xenopus* MAP kinase activator itself is a 45 kDa phosphoprotein and is inactivated by protein phosphatase 2A treatment *in vitro*. Microinjection of the purified activator into immature oocytes results in immediate activation of MAP kinase. Further experiments using microinjection as well as cell free extracts have shown that *Xenopus* MAP kinase activator is an intermediate between MPF and MAP kinase. Thus, MAP kinase activator plays a key role in the phosphorylation cascade.

Key words: MAP kinase/MAP kinase activator/MPF/phosphorylation cascade/tyrosine phosphorylation

Introduction

A group of 40-45 kDa serine/threonine kinases, termed MAP kinase, was originally described as having a serine/threonine kinase activity that is activated by insulin (Ray and Sturgill, 1987, 1988) and by many growth factors and phorbol esters (Hoshi *et al.*, 1988, 1989; Rossomando *et al.*, 1989). MAP kinases are characterized by their ability to phosphorylate microtubule associated protein 2 (MAP2) and myelin basic protein (MBP), but not histone or casein *in vitro*, and have been shown to be activated in response to various extracellular stimuli in many cell types (Pelech *et al.*, 1988; Ahn *et al.*, 1990; Ely *et al.*, 1990; Gomez *et al.*, 1990; Gotoh *et al.*, 1990a,b; Haystead *et al.*, 1990; Miyasaka *et al.*, 1990; Nel *et al.*, 1990; Sanghera *et al.*, 1990; Tsao *et al.*, 1990). Two related MAP kinases, whose apparent molecular masses are 43 kDa and 41 kDa, are commonly activated in mammalian cells (Gotoh *et al.*, 1990a, b; Ahn *et al.*, 1990; Boulton *et al.*, 1991; Boulton and Cobb, 1991). Molecular cloning of these mammalian MAP kinases has been carried out by Boulton *et al.* (1990, 1991) who named the 43 kDa and 41 kDa MAP kinases ERK1 and ERK2, respectively, for extracellular signal regulated kinases 1 and 2. MAP kinases are unique in that they are active only when both tyrosine and threonine/serine residues are phosphorylated (Anderson *et al.*, 1990). Both 43 kDa and 41 kDa MAP kinases become phosphorylated not only on tyrosine but also on threonine/serine residues after cells are treated with extracellular stimuli and dephosphorylation of either type of residue abolishes their kinases activity (Ahn *et al.*, 1990; Anderson *et al.*, 1990; Boulton and Cobb, 1991; Chung *et al.*, 1991). Payne *et al.* (1991) determined sites of phosphorylation on tyrosine and threonine residues in the active 41 kDa MAP kinase. Interestingly, these two MAP kinases were shown to phosphorylate and activate another growth factor-activated kinase, $p90^{rsk}$ (S6 kinase II) (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn and Krebs, 1990; Chung *et al.*, 1991). Thus, MAP kinase may play an important role in the phosphorylation cascade involved in the growth factor-stimulated signal transduction pathways.

Recently, MAP kinase has been shown to be activated and tyrosine phosphorylated during M phase of the *Xenopus* oocyte cell cycles (Gotoh *et al.*, 1991a; Posada *et al.*, 1991; Ferrell *et al.*, 1991). We purified and characterized this 42 kDa *Xenopus* M phase MAP kinase, isolated its cDNA clone and revealed its possible function as a regulator of microtubule dynamics (Gotoh *et al.*, 1991a,b). We have further shown that MPF ($p34^{cdc2}$-cyclin complex), a central control element in the G_2-M transition (Murray and Kirschner, 1989; Maller, 1990; Nurse, 1990), acts as an upstream activator of MAP kinase (Gotoh *et al.*, 1991b). MAP kinase may have a pivotal position not only in the growth factor signalling pathway but also in the MPF-induced phosphorylation cascade that may regulate M phase cellular events. Therefore, understanding the mechanism of activation and phosphorylation of MAP kinase is of great importance and interest.

It has been suggested that there may exist two protein kinases, a tyrosine kinase and a serine/threonine kinase, immediately upstream of MAP kinase (Anderson *et al.*, 1990). However, a recent report of Seger *et al.* (1991) has shown the ability of mammalian MAP kinases to undergo autophosphorylation on both tyrosine and threonine residues which raised the possibility that an upstream, direct activator of MAP kinase is not necessarily a kinase. Ahn *et al.* (1991) described an activity in epidermal growth factor-treated cells that is capable of inducing activation and phosphorylation of mammalian MAP kinases. Gomez and Cohen (1991) reported partial purification of the factor showing a similar activity from nerve growth factor-stimulated PC12 cells. However, as the activating factor has not been purified yet, it is uncertain whether this MAP kinase activating activity represents a direct, immediate upstream activator of MAP kinase. Thus, little is known about the identity and

biochemical properties of the activator activity or about the mechanism of the action on MAP kinase.

Since MPF is not a direct activator of *Xenopus* M phase MAP kinase (Gotoh *et al.*, 1991b), an activator of MAP kinase may exist downstream of MPF. Here, we have identified and purified a direct activator of MAP kinase in *Xenopus* mature oocytes. The purified activator is a 45 kDa protein and is able to induce phosphorylation on tyrosine and threonine/serine residues and activation of native inactive MAP kinases as well as bacterially produced *Xenopus* MAP kinase. This clearly demonstrates for the first time that one direct upstream activator is sufficient for activation of MAP kinase. Very interestingly, the activator itself is a phosphoprotein and its activity is lost upon protein phosphatase 2A treatment. Moreover, microinjection studies and the cell free system provide evidence that the MAP kinase activator is activated under the control of MPF. These results suggest that the activator identified here is a key intermediate in the MAP kinase activation pathway.

Results

Identification of a Xenopus MAP kinase activator

To search for a factor in mature *Xenopus* oocytes that is able to activate MAP kinase (i.e. a MAP kinase activator), we expressed *Xenopus* MAP kinase in *Escherichia coli*. We produced the recombinant MAP kinase whose molecular mass was slightly larger than that of native enzyme by introducing 10 amino acid residues in the N-terminus to distinguish them after SDS−polyacrylamide gel electrophoresis (Figure 1). Sequencing of N-terminal amino acids and immunoblotting with anti-MAP kinase antibodies of purified recombinant MAP kinase confirmed its identity (Figure 1, lanes 1 and 3, ▷). The recombinant MAP kinase preparation purified from the inclusion body contained an additional polypeptide that was identified as an N-terminal truncated form of MAP kinase by its N-terminal amino acid sequencing and reactivity to anti-MAP kinase antibodies (Figure 1, lanes 1 and 3, ◇). Recombinant MAP kinase purified from soluble extracts of the *E.coli* contained no truncated forms (Figure 1, lanes 2 and 4). As these two preparations of full-length (▷) recombinant MAP kinases were qualitatively indistinguishable in their reactivity to *Xenopus* MAP kinase activator, in this paper we show only data obtained with the recombinant MAP kinase preparation purified from the inclusion body.

We found that the recombinant MAP kinase can be activated *in vitro* by incubation with an M phase extract of mature *Xenopus* oocytes in the presence of ATP. In our kinase detection assay within polyacrylamide gels containing MBP after SDS−PAGE (Gotoh *et al.*, 1990a,b), the recombinant MAP kinase was virtually undetectable because of its very low kinase activity, while after the incubation it was activated dramatically and became easily detectable in the gel (data not shown). To identify a MAP kinase activator activity, we fractionated the M phase extract by DEAE−cellulose chromatography. Each fraction was incubated with or without recombinant MAP kinase and ATP, and then each mixture was subjected to a kinase detection assay within gels. The activity to activate the MBP kinase activity of recombinant MAP kinase was mostly recovered in the flow-through fraction (Figure 2A), separate from endogenous MAP kinase which was eluted between 0.1 and 0.3 M NaCl (Figure 2A).

This MAP kinase activator activity was undetectable in an extract of immature oocytes, but increased dramatically during progesterone-induced oocyte maturation (Figure 2B). This increase was accompanied by MAP kinase activation (Figure 2B). Upon fertilization, a high activator activity in metaphase II dropped rapidly and became undetectable within 30 min (Figure 2C). The decrease in activator activity was followed by a decrease in MAP kinase activity (Figure 2C). Thus, the activity of the activator oscillated depending on the cell cycle status and this activator may be qualified as an *in vivo* activator of MAP kinase.

Purification of Xenopus MAP kinase activator

We purified a *Xenopus* MAP kinase activator by sequential chromatography on DEAE−cellulose, heparin−Sepharose, phosphocellulose, hydroxylapatite and Mono-S (Figure 3 and Table I). For each type of chromatography, the activity that enhanced MBP kinase activity of recombinant MAP kinase was eluted as a single peak and was completely coincident with the major peak of activity that induced phosphorylation of the full-length recombinant MAP kinase (data not shown). Performing chromatography on hydroxylapatite (data not shown) and Mono-S, the elution of a 45 kDa protein coincided completely with that of the activator activity, namely, enhancement of the MBP kinase activity and phosphorylation of the recombinant MAP kinase (Figure 3B). Essentially no kinase activity toward MBP was detected in the activator fraction alone (Figure 3B, ○). The activator (45 kDa protein) was purified ~1300-fold from soluble extracts of mature oocytes, as the purification was ~650-fold from the DEAE−cellulose fraction (Table I) and ~50% of proteins in crude extracts were recovered in the flow-through

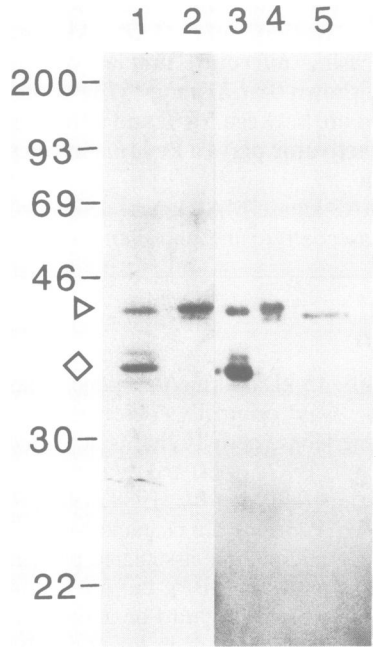

Fig. 1. Recombinant *Xenopus* MAP kinase. Recombinant *Xenopus* MAP kinase was purified from the inclusion body fraction (lanes 1 and 3) or from the soluble fraction (lanes 2 and 4) as described in Materials and methods. Lanes 1 and 2: each sample (0.6 μg of protein) was electrophoresed in a 12% acrylamide gel and stained with Coomassie blue. Lanes 3 and 4: each sample (18 ng of protein) was immunoblotted with anti-MAP kinase antiserum (DME). As a control, *Xenopus* M phase MAP kinase purified as described (Gotoh *et al.*, 1991a) was also immunoblotted (lane 5).

of DEAE−cellulose column (data not shown). The final preparation was >95% pure (Figure 3A). It should be noted that the N-terminal truncated form of recombinant MAP kinase (see Figure 1, lanes 1 and 3, ◇) was not phosphorylated or activated at all upon incubation with the activator and ATP (Figure 3B, middle panel, ◇).

MAP kinase activator is a phosphoprotein and is inactivated by protein phosphatase 2A treatment

As MAP kinase is activated under the control of MPF (Gotoh *et al.*, 1991b), it is quite possible that MAP kinase activator is also activated by phosphorylation *in vivo*. To test this possibility, we treated the purified activator with protein phosphatase 2A. This treatment completely inactivated the ability of the activator to induce phosphorylation of recombinant MAP kinase and to activate its MBP kinase activity (Figure 4). In control experiments where the activity of protein phosphatase 2A was inhibited in the presence of okadaic acid during the treatment, there was no loss in activity of the activator (Figure 4). Thus, phosphorylation of the activator is required for its activity and the activator must be phosphorylated in mature oocytes.

To confirm this, we purified the activator from ^{32}P-labelled mature oocytes by sequential chromatography. The elution profiles of phosphorylated proteins (upper panel) and the activator activity (lower panel) in the final two steps, hydroxylapatite and phosphocellulose columns, are shown in Figure 5. Using both types of chromatography, the elution of the activator activity was completely coincident with that of the 45 kDa phosphoprotein. In addition, in the phosphocellulose chromatography the 45 kDa protein was the only detectable phosphorylated protein in the active fractions. These results, together with the previous purification data, indicate that *Xenopus* MAP kinase activator is a 45 kDa phosphoprotein.

Activation by MAP kinase activator of inactive MAP kinase isolated from immature oocytes

We isolated an inactive form of MAP kinase from immature *Xenopus* oocytes to examine whether the activator can act on native MAP kinase as well as bacterially expressed MAP kinase. We have previously shown that MAP kinase is present as an inactive, unphosphorylated form in immature *Xenopus* oocytes (Gotoh *et al.*, 1991a,b). Partial purification of inactive MAP kinase was achieved by sequential chromatography on DEAE−cellulose, phenyl−Sepharose, polylysine−agarose, hydroxylapatite and heparin−Sepharose (Figure 6A). Incubation of the partially purified inactive MAP kinase with the purified activator in the presence of ATP resulted in MAP kinase phosphorylation (Figure 6B, left) and kinase activation, as measured by the kinase detection assay within gels (Figure 6B, middle) and by the ordinary test tube assay (Figure 6B, right).

Mechanism of action of MAP kinase activator

We characterized the mode of action of purified MAP kinase activator on recombinant MAP kinase. As was observed for mammalian MAP kinase (Seger *et al.*, 1991), recombinant *Xenopus* MAP kinase had the ability, although very low, to undergo autophosphorylation (Figure 7A, right) and had a very weak kinase activity toward MBP. Phosphorylation of the MAP kinase and enhancement of its MBP kinase

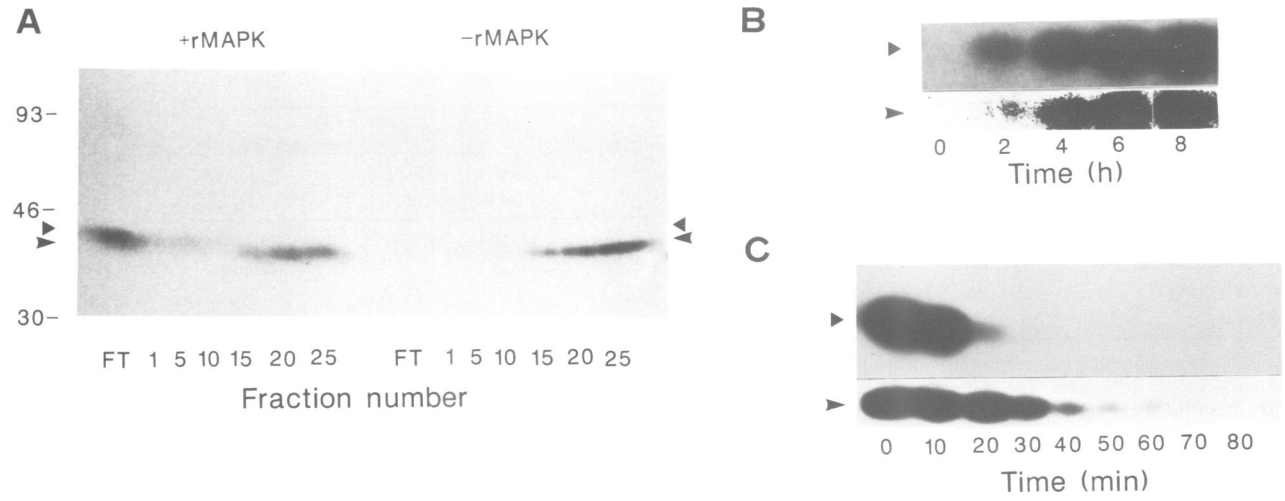

Fig. 2. Identification and cell cycle-dependent regulation of the MAP kinase activator activity. (**A**) Extracts obtained from 0.3 ml *Xenopus* mature oocytes were loaded onto a DEAE−cellulose (2 ml) column. The unadsorbed proteins were collected by washing the column with 6 ml of the equilibrating buffer and pooled as the flow-through (FT) fraction (total 12 ml). Then, the adsorbed proteins were eluted with a 40 ml linear gradient of 0−0.4 M NaCl (fraction number 1−40). An aliquot of each fraction was incubated at 25°C for 30 min with (+ rMAPK) or without (− rMAPK) recombinant MAP kinase in the presence of 50 μM ATP and 10 mM $MgCl_2$. Each sample was then subjected to the kinase detection assay within a polyacrylamide gel containing 0.5 mg/ml MBP (Gotoh *et al.*, 1990b). Closed triangles and arrowheads indicate activities of activated recombinant MAP kinase and endogenous MAP kinase, respectively. More than 95% of the activity required to activate the recombinant MAP kinase was recovered in the flow-through fraction. (**B**) Changes in activities of MAP kinase activator (upper) and MAP kinase (lower) during maturation. Immature *Xenopus* oocytes (stage VI) were incubated with 10 μM progesterone for indicated times and the extracts obtained were fractionated by DEAE−cellulose chromatography. The flow-through fraction was mixed and incubated with recombinant MAP kinase as in (A) and subjected to the kinase detection assay within a gel containing MBP. Thus, the activity of MAP kinase activator is expressed as the MBP kinase activity of recombinant MAP kinase within the gel (upper panel, a closed triangle). Endogenous MAP kinase activity was measured by the kinase detection assay within a different gel containing MBP, using the adsorbed fraction (lower panel, an arrowhead). (**C**) Changes in activities of MAP kinase activator (upper) and MAP kinase (lower) after fertilization. Mature *Xenopus* oocytes were fertilized and the extracts were obtained 0−80 min after fertilization. Activities of MAP kinase activator and MAP kinase were measured as in (B).

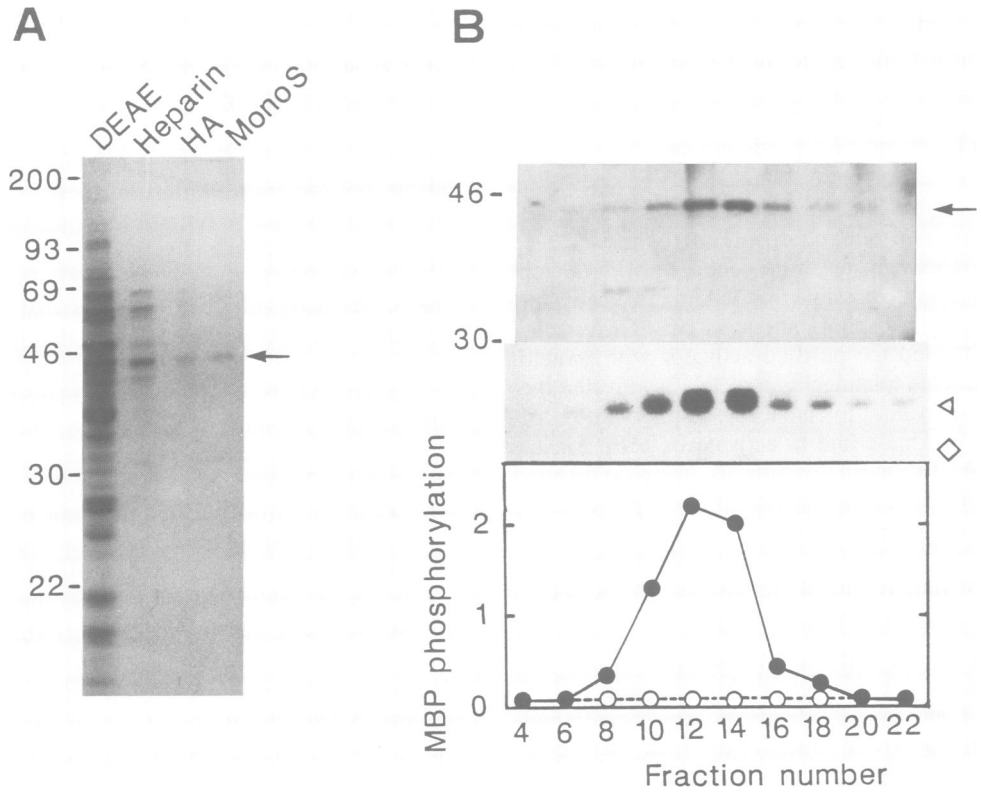

Fig. 3. Purification of *Xenopus* M phase MAP kinase activator. (**A**) *Xenopus* M phase MAP kinase activator was purified from mature oocytes as described in Materials and methods. Peak fractions from chromatography on DEAE−cellulose (27.2 μg), heparin−Sepharose (1.76 μg), hydroxylapatite (0.12 μg) and Mono-S (0.08 μg) were electrophoresed on a 12% acrylamide gel and stained with Coomassie blue. The arrow denotes the activator. (**B**) Chromatography on Mono-S. Coomassie blue staining (top), phosphorylation of recombinant MAP kinase (middle) and elution profile of MBP kinase activity in the presence (closed circles) and absence (open circles) of recombinant MAP kinase. In this chromatography, the adsorbed proteins were eluted with a 15 ml linear 0−400 mM NaCl gradient (fraction number 1−30). The electrophoretic positions of the full-length (◁) and truncated (◇) recombinant MAP kinases are shown. The arrow denotes the activator. The MBP phosphorylation is expressed in an arbitrary unit.

Table I. Purification of *Xenopus* MAP kinase activator

Step	Total protein (mg)	Total activity[a] (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
DEAE−cellulose	1190	12.7	0.011	100	1
Heparin−Sepharose	24.2	5.7	0.24	45	22
Hydroxyl−apatite	0.75	0.97	1.3	7.6	121
Mono S	0.03	0.21	7	1.6	652

[a]The activator activity of each fraction was defined as the MBP phosphorylating activity of recombinant MAP kinase activated by the fraction under our standard assay conditions. The MBP phosphorylation was determined by the kinase detection assay within gels containing MBP. The bands were excised from the dried gel and quantified by Cerenkov counting.

activity were both dependent on the concentration of the activator added (data not shown). For example, at 10 μg/ml activator the MBP kinase activity of MAP kinase was >100-fold higher than that in the absence of the activator. ATP was absolutely required for activation of recombinant MAP kinase by the activator. GTP could not substitute for ATP (data not shown). Thus, ATP dependent phosphorylation of MAP kinase may be necessary for its activation.

To judge whether recombinant MAP kinase is activated correctly by the activator *in vitro*, we compared the substrate recognition of the *in vitro* activated MAP kinase with that of authentic MAP kinase purified from mature oocytes (Gotoh *et al.*, 1991a). Both kinases phosphorylated MBP and MAP2 very well, but not histone or casein (data not shown). Furthermore, both kinases phosphorylated MBP exclusively on a threonine residue (data not shown) and the one-dimensional phosphopeptide mapping patterns of MAP2 phosphorylated by each of the kinases were indistinguishable from each other (data not shown). Thus, both kinases exhibited the same specificity of the substrate and site recognition, suggesting that the activator can activate MAP kinase correctly.

To characterize the interaction of the activator with recombinant MAP kinase in more detail, we followed the time course of phosphorylation and activation of the MAP kinase during incubation with ATP in the presence and absence of the activator (Figure 7). In the absence of the activator autophosphorylation of MAP kinase was very slow but detectable (Figure 7A) and occurred mainly on tyrosine and to some extent threonine residues (Figure 7D). After

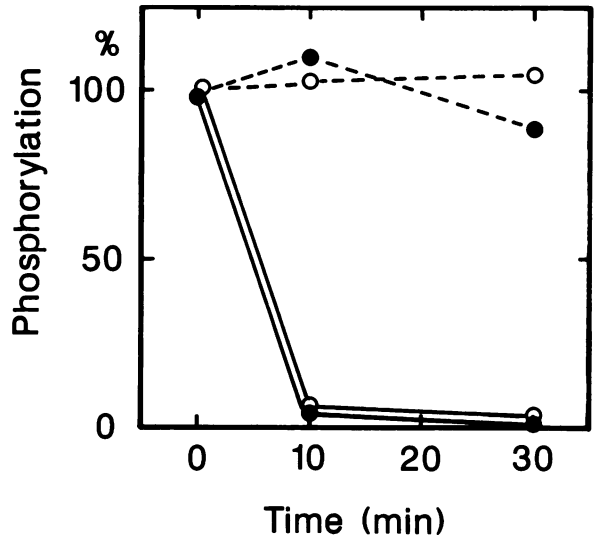

Fig. 4. Deactivation of MAP kinase activator by phosphatase 2A. The peak fraction (no. 12) from chromatography on Mono-S was dialysed and then incubated for the indicated times at 30°C with the catalytic subunit of phosphatase 2A (1 μg/ml) as described in Materials and methods. The samples were then immediately assayed for the activator activity. Phosphorylation of recombinant MAP kinase, open circles; MBP kinase activity of recombinant MAP kinase, closed circles. In control incubations, okadaic acid was added simultaneously with phosphatase 2A (broken lines).

a 200 min incubation, a slowly migrating species appeared (Figure 7A) that was phosphorylated mainly on tyrosine and serine residues (Figure 7D). But, in the kinase detection assay within polyacrylamide gels, no kinase activity toward MBP was seen (Figure 7C).

In contrast, in the presence of the activator, phosphorylation of MAP kinase was much faster (Figure 7A). An electrophoretically retarded band appeared very rapidly (Figure 7A,) and coincidentally the kinase activity of MAP kinase became detected within 3 min of incubation and was maximally activated within 60 min (Figure 7C). The activator itself became phosphorylated by the activated MAP kinase after longer incubations (Figure 7A, arrow). The electrophoretical position of the active MAP kinase polypeptide (Figure 7C) was identical to that of the electrophoretically retarded (upper) band (Figure 7A). Furthermore, the kinase detection assay following re-electrophoresis of samples excised from the upper and lower bands confirmed that the upper band has the kinase activity (data not shown). Phosphorylation of the lower band occurred mainly on tyrosine and to some extent threonine residues (Figure 7D), as was seen in the absence of the activator, while the upper band (active species) was phosphorylated on tyrosine and threonine residues evenly and additionally on serine residues (Figure 7D). The anti-phosphotyrosine antibody immunoblot confirmed the tyrosine phosphorylation of both bands (Figure 7B). It is noted that autophosphorylation of native active MAP kinase purified from *Xenopus* eggs occurs mainly on serine (data not shown).

In summary, the fully active form of MAP kinase can be produced only by the action of the activator. In other words, the activator is not a mere accelerator of the autophosphorylation of MAP kinase. However, the purified activator did not exhibit any kinase activity toward MBP, MAP2, histone or casein (data not shown) and it did not phosphorylate the truncated recombinant MAP kinase (Figure 3B, ◇) which contained presumable tyrosine and threonine phosphorylation sites that were determined by Payne *et al.* (1991) for mammalian MAP kinase. As is consistent with the knowledge that the truncated recombinant MAP kinase lacks part of the kinase domain, it did not undergo autophosphorylation (data not shown). Therefore, even the activator induced phosphorylation of MAP kinase seems to be dependent on integrity of the kinase domain of MAP kinase. We hypothesize now that MAP kinase activator may induce a qualitative change in the activity of inactive MAP kinase to undergo autophosphorylation by binding to it and changing its conformation and thus, MAP kinase may be capable of changing into an active form in the presence of ATP and activator.

Activation of MAP kinase by MAP kinase activator in immature oocytes and in cell free extracts

To examine whether the activator can indeed activate MAP kinase *in vivo*, we first tested the ability of the purified activator to induce activation of MAP kinase in an immature oocyte extract, which was diluted with a buffer solution containing phosphatase inhibitors. The purified activator, when added to the extract, induced activation of MAP kinase in a time and concentration dependent manner (data not shown). Then, we microinjected the purified activator into immature oocytes. MAP kinase activity, as measured by the kinase detection assay within gels, increased within 5 min of the microinjection, peaked at 10 min and then decreased gradually (Figure 8A). Microinjection of the buffer solution alone did not induce activation of MAP kinase (data not shown).

We further found that the addition of the purified activator into cell free extracts prepared without dilution or phosphatase inhibitors from *Xenopus* interphase eggs resulted in marked activation of MAP kinase (Figure 8B). These results suggest that the active MAP kinase activator alone may be sufficient for MAP kinase activation *in vivo*.

Activation of MAP kinase activator by MPF

We have previously shown that MPF is an upstream, indirect activator of MAP kinase (Gotoh *et al.*, 1991b). Therefore, it is of crucial importance to know whether MAP kinase activator is activated by MPF. For this we carried out microinjection of purified MPF into immature oocytes. We measured the MAP kinase activator activity as well as MAP kinase activity of the microinjected oocytes after we partially purified the activator and MAP kinase from the oocyte extracts by DEAE−cellulose chromatography as described (see Figure 2). After microinjection of MPF, the activator activity increased dramatically and MAP kinase activity increased approximately in parallel (Figure 9A). This result strongly indicates that MAP kinase activator as well as MAP kinase can be activated by MPF.

In vitro also, MPF could induce activation of MAP kinase activator as well as MAP kinase. As shown in Figure 9B, incubation of interphase extracts prepared from fertilized eggs with purified MPF resulted in a time dependent increase in both MAP kinase and activator activities. Activation of MAP kinase lagged slightly behind that of the activator. Thus, this *in vitro* system reveals the M phase phosphorylation cascade of MPF, MAP kinase activator and MAP kinase.

As purified activator is inactivated by dephosphorylation

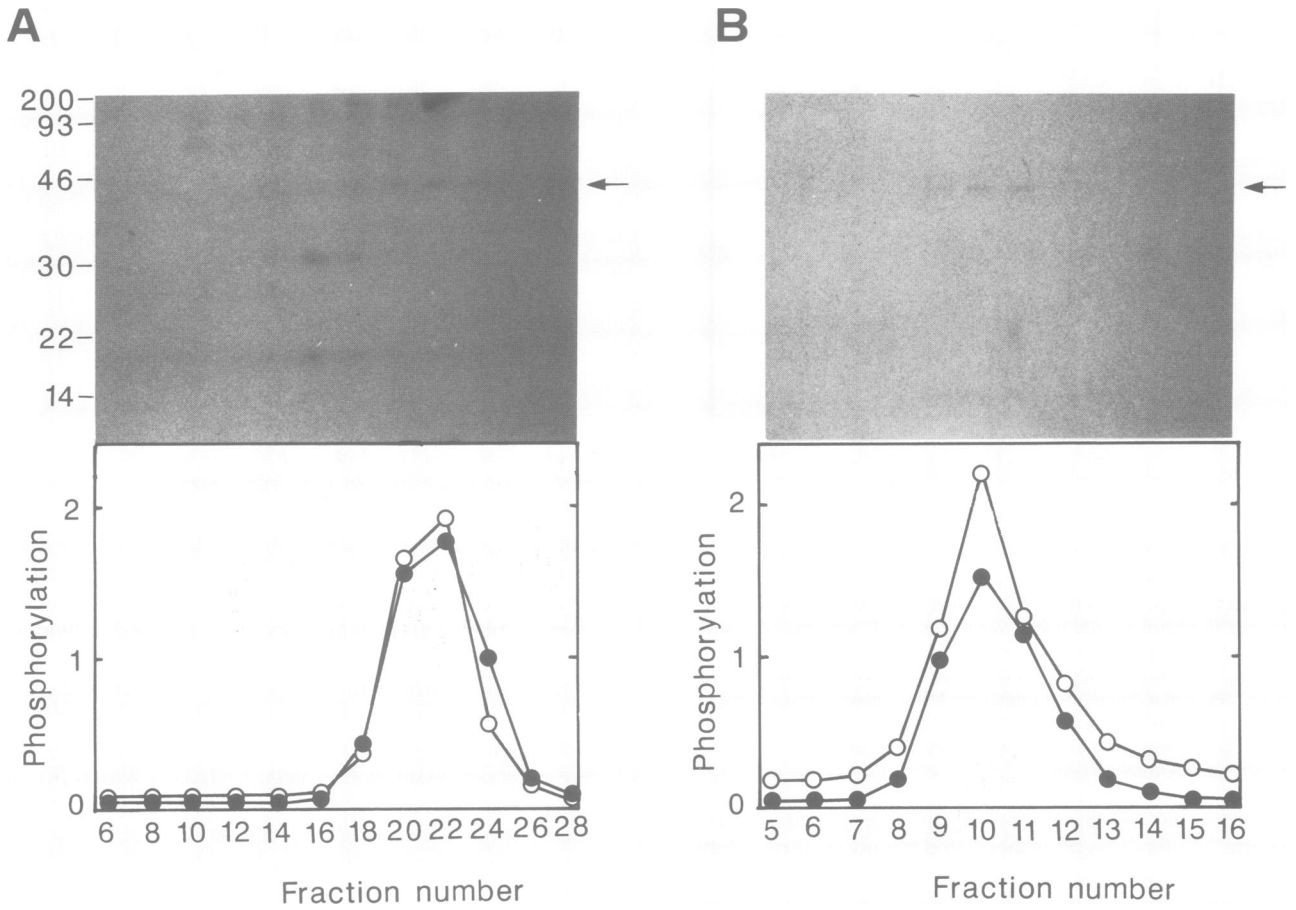

Fig. 5. MAP kinase activator is a phosphoprotein. MAP kinase activator was purified from an extract prepared from ^{32}P-labelled mature oocytes by sequential chromatography on DEAE−cellulose, heparin−Sepharose, hydroxylapatite and phosphocellulose as described in Materials and methods. (**A**) Chromatography on hydroxylapatite. Numbered fractions were electrophoresed in a 14% acrylamide gel which was exposed to X-ray film for 18 days (top). Elution profiles of the ability of the activator to phosphorylate recombinant MAP kinase (open circles) and to enhance the MBP kinase activity of recombinant MAP kinase (closed circles) are shown (bottom). Both activities are expressed as arbitrary units. In this chromatography system, the adsorbed proteins were eluted with a 60 ml linear 30−200 mM potassium phosphate gradient (fraction number 1−40). (**B**) Chromatography on phosphocellulose in which the adsorbed proteins were eluted with a 30 ml linear 0−250 mM NaCl gradient (fraction number 1−20). The autoradiography (top) and the activator activity (bottom) are shown as in (A). The arrow indicates the 45 kDa phosphoprotein.

(Figure 4), we tried to reactivate the MAP kinase activator by MPF. However, purified MPF could not reactivate the activator previously inactivated by phosphatase 2A treatment (data not shown). It is likely, therefore, that there may exist an intermediate kinase(s) between MPF and MAP kinase activator.

Discussion

A previous study has shown that purified MPF can activate MAP kinase when microinjected into immature oocytes or added to the cell free extracts obtained from interphase eggs, but MPF cannot directly reactivate MAP kinase previously inactivated by phosphatase 2A treatment (Gotoh *et al.*, 1991b). Therefore, there must exist an intermediate(s) between MPF and MAP kinase, that is, MPF is an indirect activator of MAP kinase. Here, we have identified and purified a protein factor (MAP kinase activator) from *Xenopus* mature oocytes that is capable of directly inducing activation and phosphorylation of bacterially expressed, inactive MAP kinase. The activator activity was eluted as a single peak in five different column chromatographies and has been purified to near homogeneity after these steps. Several lines of evidence suggest that the activator identified here is a true *in vivo* activator of M phase MAP kinase in *Xenopus* oocytes. First, as is consistent with its *in vivo* role, the activator activity increases in parallel with, or slightly preceding, activation of MAP kinase during maturation (Figure 2B) and decreases slightly faster than inactivation of MAP kinase during exit from M phase (Figure 2C). Second, the purified activator can act on native inactive MAP kinase (Figure 6) as well as bacterially produced MAP kinase. Third, MAP kinase that has been activated by the purified activator is indistinguishable from authentic active MAP kinase purified from *Xenopus* mature oocytes in terms of their substrate and site recognition properties. Fourth, the purified activator can activate endogenous inactive MAP kinase when microinjected into immature oocytes or added to extracts prepared from the interphase eggs or immature oocytes (Figure 8). These results indicate that activation of MAP kinase activator alone may be sufficient for activation of MAP kinase in cells. Fifth, the activator activity can be activated by microinjection of MPF into immature oocytes or by incubation of MPF with extracts prepared from interphase cells (Figure 9), suggesting that the activator may be an intermediate between MPF and MAP kinase. Sixth, as expected from its assumed position in the phosphorylation cascade, *Xenopus* MAP kinase activator is a phosphorylated

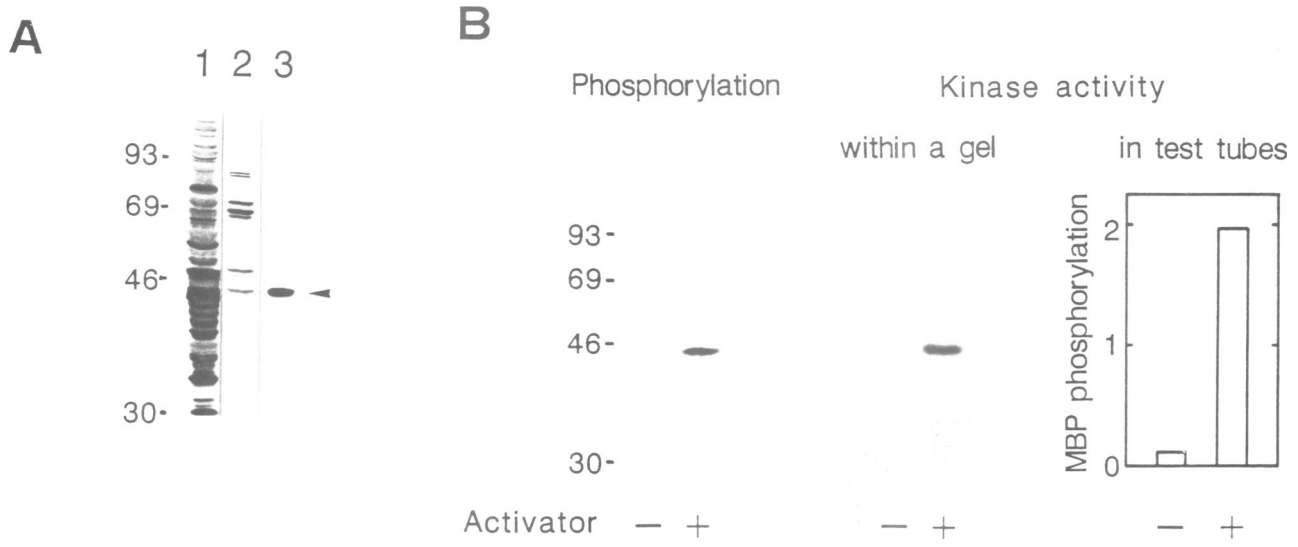

Fig. 6. Activation of native inactive MAP kinase by MAP kinase activator. (**A**) Purification of native inactive MAP kinase from *Xenopus* immature oocytes. Silver staining of crude extract prepared from immature oocytes (lane 1) and partially purified inactive MAP kinase (lane 2). Immunoblotting of the partially purified fraction with anti-MAP kinase antibody (DME, lane 3). The arrowhead denotes the inactive MAP kinase. (**B**) Activation of native inactive MAP kinase by the purified activator. Partially purified inactive MAP kinase was incubated for 20 min at 30°C without (−) or with (+) the purified activator (5 μg/ml) and ATP (50 μM) under the standard conditions. The left panel shows the phosphorylation of MAP kinase. The middle panel shows the kinase detection assay within a gel containing MBP. The right panel shows the test tube assays for MBP phosphorylating activity (an arbitrary unit).

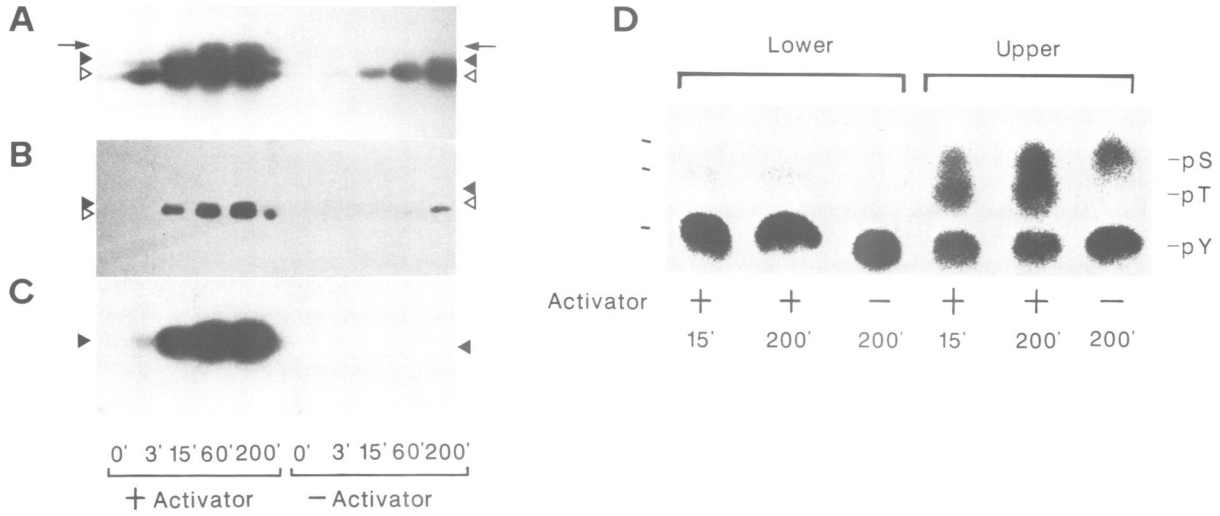

Fig. 7. Time course of phosphorylation and activation of recombinant MAP kinase by MAP kinase activator. (**A**) Recombinant MAP kinase (30 μg/ml) was incubated with [γ-^{32}P]ATP in the presence (left, +) or absence (right, −) of MAP kinase activator (the peak fraction of Mono-S, 7 μg/ml) as described in Materials and methods for indicated times. The samples were then electrophoresed in a 12% acrylamide gel, which was exposed to X-ray film. Arrows, closed triangles and open triangles denote the electrophoretical positions of the activator, the mobility-shifted recombinant MAP kinase (upper band) and the unshifted recombinant MAP kinase (lower band), respectively. (**B**) The reactions were carried out as in (A) except thar radiolabelled ATP was omitted. The samples were immunoblotted with anti-phosphotyrosine antibody (PY20, ICN). (**C**) The samples obtained as in (B) were electrophoresed in a 12% acrylamide gel containing 0.5 mg/ml MBP, and the kinase detection assay within gels was carried out as described (Gotoh *et al.*, 1990b). (**D**) Phosphoamino acid analyses of the phosphorylated bands in (A).

protein (a 45 kDa phosphoprotein) and its activity is completely inactivated by dephosphorylation (Figures 3, 4 and 5). Taken together, these results strongly indicate that *Xenopus* MAP kinase activator is a key intermediate in the MAP kinase activation pathway.

This study reveals that the activator is a single component. This fact apparently contradicts the previous suggestion of Anderson *et al.* (1990) that MAP kinase would be activated at a point of convergence between tyrosine and serine/threonine phosphorylation pathways, although their original observation that MAP kinase is active only when both tyrosine and threonine/serine residues are phosphorylated has been confirmed here. In fact, our purified activator induces phosphorylation of both tyrosine and threonine/serine residues of inactive MAP kinase and thereby induces its full activation as a kinase. Two results should be considered in elucidating the mechanism of the action of MAP kinase activator. First, the inactive *Xenopus* MAP kinase had an ability to autophosphorylate itself mainly on tyrosine and to some extent threonine residues, as previously described for mammalian MAP kinase (Seger *et al.*, 1991). Second, the activator not only potently enhanced tyrosine

phosphorylation of inactive MAP kinase but also induced its threonine phosphorylation dramatically. Thus, the activator may be assumed to be a kinase with specificity for both tyrosine and serine/threonine, or a binding factor that changes the ability of inactive MAP kinase to undergo autophosphorylation. The latter seems to be more plausible because little or no kinase activity toward exogenous substrates except for MAP kinase was detected in the purified activator. Furthermore, only the full length, inactive MAP kinase was phosphorylated by the action of the activator and the truncated form of inactive MAP kinase, which lacks part of the kinase domain but contains presumable phosphorylation sites (Payne *et al.*, 1991), was not phosphorylated at all by the activator. This suggests that the kinase activity of MAP kinase may be a prerequisite for its activation by the activator. However, it is still possible that the activator is a kinase with a very strict, narrow substrate specificity. For example, the activator might be a kinase that phosphorylates at threonine/serine only in the tyrosine phosphorylated form of inactive MAP kinase. In this case, it is assumed that the threonine/serine phosphorylation site becomes recognizable by the activator only after the initial autophosphorylation of MAP kinase on tyrosine. This possibility can explain the necessity of the kinase domain of MAP kinase for its activation by the activator, but is not easily compatible with the observation that tyrosine phosphorylation is greatly promoted by the activator. In any case, the present finding that the activator is a single protein indicates that MAP kinase activation *in vivo* does not necessarily require two or more upstream signalling pathways, but needs only one upstream activating pathway.

An electrophoretical mobility shift of *Xenopus* MAP kinase occurs when it becomes active during maturation (Gotoh *et al.*, 1991b). The MAP kinase activator can induce a mobility shift of recombinant inactive MAP kinase *in vitro*. The activator induced upper band (electrophoretically retarded band) of MAP kinase alone may be responsible for the activated kinase activity. Phosphorylation of the lower band occurred mainly on tyrosine residues, while phosphorylation of the upper band occurred on serine/threonine/tyrosine residues. Thus, tyrosine phosphorylation of MAP kinase alone may not be sufficient for its activation and electrophoretical mobility shift. In the absence of the activator, the prolonged autophosphorylation of MAP kinase resulted in an electrophoretical mobility shift. This autophosphorylated upper band is not equivalent to the activator induced upper band, since the autophosphorylated upper band had no kinase activity and the phosphorylation occurred mainly on tyrosine and serine. Further, the activator induced upper band migrated slightly more slowly than the autophosphorylation upper band in the SDS−PAGE (unpublished data). Therefore, the activator may not act as a mere accelerator of autophosphorylation of inactive MAP kinase, but can induce changes in the quality of phosphorylation.

To examine whether mammalian mitogen activated MAP kinase activator can substitute for the *Xenopus* M phase activated activator, we have partially purified the EGF activated MAP kinase activator (K.Shirakabe, Y.Gotoh and E.Nishida, unpublished data). Its behaviour on three different chromatography columns was quite similar to that of the M phase *Xenopus* MAP kinase activator described here. The EGF activated MAP kinase activator could activate inactive *Xenopus* MAP kinase as well as bacterially expressed ERK1

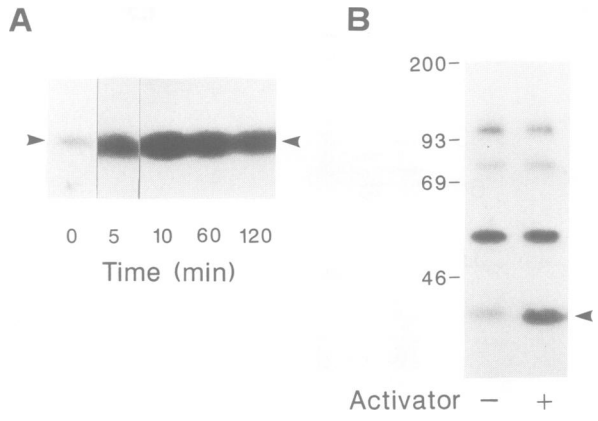

Fig. 8. Purified MAP kinase activator activates MAP kinase in immature *Xenopus* oocytes (**A**) and in interphase extracts (**B**). (A) Purified *Xenopus* MAP kinase activator was microinjected into immature *Xenopus* oocytes (stage VI). Various times after the microinjection the oocytes were homogenized and the MAP kinase activity (marked by an arrowhead) in the oocytes was measured by the kinase detection assay within MBP-containing gels. (B) Interphase extracts prepared as described previously (Gotoh *et al.*, 1991b) from fertilized *Xenopus* eggs were incubated with or without purified *Xenopus* MAP kinase activator (10 μg/ml) at 25°C for 15 min. After the reaction was stopped by the addition of SDS buffer, the kinase activity of the whole extract was measured by the kinase detection assay within a gel containing MBP. The arrowhead indicates the 42 kDa MAP kinase.

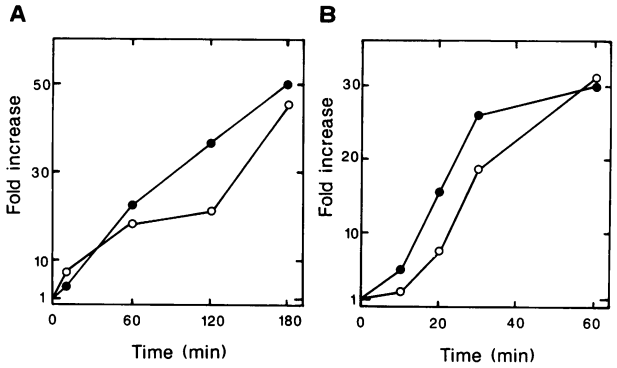

Fig. 9. MPF activates MAP kinase activator and MAP kinase in immature oocytes (**A**) and in interphase extracts (**B**). (A) Purified MPF prepared from *Xenopus* mature oocytes was microinjected into immature oocytes (stage VI). Various times after the microinjection the oocyte extract obtained was subjected to DEAE−cellulose chromatography and activities of MAP kinase activator (closed circles) and MAP kinase (open circles) were determined by the kinase detection assay within gels containing MBP as described in Figure 2B. The activities were quantified by counting the radioactivity of the corresponding position of the polyacrylamide gel. The amount of MPF microinjected into the oocyte corresponded to ~5% of that in the mature oocyte, as determined by measuring the histone H1 kinase activity. (B) Interphase extracts prepared from fertilized *Xenopus* eggs were incubated with purified MPF at 25°C for various times. Then, both activities of MAP kinase activator (closed circles) and MAP kinase (open circles) were measured as in (A) and plotted. The amount of MPF added to the extracts corresponded to 20% of that in metaphase II extracts, as determined by measuring the histone H1 kinase activity.

(generously provided by Steven Pelech). Moreover, the *Xenopus* M phase MAP kinase activator could activate the recombinant ERK1 (unpublished data). Thus, the interaction between MAP kinase and the activator may not be specific to species or phase of the cell cycle. The activator may be a very conservative protein throughout evolution, like MAP

kinase. The signal transduction systems in G_0/G_1 and M phases may share the same phosphorylation cascade involving MAP kinase and its activator.

As described before, the present study has revealed that *Xenopus* MAP kinase activator functions as an intermediate between MPF and MAP kinase and that the activator may be activated by phosphorylation. However, purified MPF could not reactivate the activator previously inactivated by protein phosphatase 2A treatment. Therefore, an intermediate kinase may exist between MPF and the activator. In *Xenopus* oocytes, S6 kinase II is reported to be activated by progesterone treatment (Maller, 1990). Dephosphorylated S6 kinase II could be phosphorylated and reactivated by MAP kinase *in vitro* (Sturgill *et al.*, 1988). Taken together, there could be a phosphorylation cascade triggered by MPF (MPF → → MAP kinase activator → MAP kinase → S6 kinase II) which may contribute to execution of cell reorganization at M phase, including microtubule rearrangement that is suggested to be regulated at least in part by MAP kinase (Gotoh *et al.*, 1991a).

Materials and methods

Production and purification of recombinant MAP kinase

The full-length cDNA encoding *Xenopus* MAP kinase has been described previously (Gotoh *et al.*, 1991b). The *Eco*RI site was introduced immediately upstream of the translation initiator ATG by PCR. A 1.2 kb *Eco*RI−*Kpn*I fragment of MAP kinase cDNA was inserted between the *Eco*RI and *Kpn*I sites of pUC18 in which 12 bp (5′-GCCAAGCTCCGG-3′) was inserted just prior to the *Eco*RI site. This plasmid produces a fusion protein that contains 10 additional amino acids (TMITPSSGNS) on the authentic N-terminus of MAP kinase. This plasmid was transformed into MV1184 and grown to an OD_{600} of 0.3 at 37°C in 4 l of L-broth. After a 12 h incubation in the presence of 1 mM IPTG, bacteria were collected and suspended in 20 ml of 10 mM Tris−Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA to which 1 mg/ml lysozyme was then added. After incubation for 30 min, the suspension was frozen, thawed and mixed with 200 ml of 20 mM Tris−Cl, pH 7.5, 3 mM $MgCl_2$, 1 mM $MnCl_2$, 1% NP40, 1 mM PMSF, 1 mM dithiothreitol and 30 μg/ml DNase I, and homogenized. After incubation at 30°C for 30 min, the inclusion body fraction was collected by centrifugation for 30 min at 15 000 r.p.m. with an RPR 16 rotor (Hitachi). The supernatant was taken as the soluble fraction and the pellet was washed once in 100 ml of 0.5% Triton X-100, 10 mM EDTA, 100 mM NaCl and 50 mM Tris−Cl, pH 7.5. The washed inclusion body fraction was dissolved in 20 ml of 8 M urea, 50 mM Tris−Cl, pH 7.5, 1 mM EGTA, 50 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF and mixed with 60 ml of PS buffer (20 mM Tris−Cl, pH 7.5, 2 mM EGTA, 1 mM PMSF, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate). After centrifugation, the supernatant was loaded onto a phenyl−Sepharose (30 ml) column equilibrated with PS buffer. After washing the column, proteins were eluted with a 400 ml linear 0−1.5% Brij 35 gradient. The elution of recombinant MAP kinase was judged by ELISA with the anti-MAP kinase antiserum (DME) against the near C-terminal 14 amino acid sequence (DMELDDLPKERLKE) of ERK1, whose production and specificity were described previously (Gotoh *et al.*, 1991b). The peak fractions (eluting at 0.25−0.60% Brij 35) were pooled and loaded onto a polylysine−agarose (10 ml) column equilibrated with PS buffer. After washing the column, proteins were eluted with a 200 ml linear 0−0.8 M NaCl gradient. The peak fractions (eluting at 0.3−0.4 M NaCl) were pooled and stored at −80°C. From the soluble fraction of the bacteria, recombinant MAP kinase was also purified by sequential chromatography on DEAE−cellulose, phenyl−Sepharose and polylysine−agarose essentially as described previously (Gotoh *et al.*, 1990b). The elution of recombinant MAP kinase from these columns was slightly different from that of native active MAP kinase. Sequencing of N-terminal amino acids and immunoblotting with two anti-MAP kinase antibodies, raised against the C-terminal and the near C-terminal 14 amino acid sequences of ERK1, respectively, revealed that the 44 kDa recombinant MAP kinase prepared from both the soluble and the inclusion body fractions contained the complete sequence of MAP kinase and an additional N-terminal sequence described above and that the truncated (35 kDa) recombinant MAP kinase, prepared from the inclusion body fractions, starts at Leu78 and therefore lacks the ATP binding site (Hanks *et al.*, 1988). The antiserum against the C-terminal 14 amino acid sequence (QETARFQPGAPEAP), termed QET, was produced by the method described previously for the antiserum against the near C-terminal sequence, termed DME (Gotoh *et al.*, 1991b).

Purification of Xenopus MAP kinase activator

Extracts of mature *Xenopus* oocytes were prepared as described by Gotoh *et al.* (1991a) and dialysed against buffer A (20 mM Tris−HCl, pH 8.0, 20 mM β-glycerophosphate, 2 mM EGTA, 1 mM PMSF, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate). The dialysate (~1800 mg of protein, representing 100 ml of dejellied eggs) was loaded onto a DEAE−cellulose (60 ml) column equilibrated with buffer A. The column was washed with 30 ml of buffer A and the flow-through fractions were loaded onto a heparin−Sepharose (15 ml) column equilibrated with buffer B (20 mM Tris−Cl, pH 7.0, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1 mM PMSF, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate). After washing the column, proteins were eluted with a 450 ml linear 0−400 mM NaCl gradient. The active fractions, as detected by phosphorylation and activation of recombinant MAP kinase (see below), eluting at 80−130 mM NaCl were pooled and loaded onto a phosphocellulose (7 ml) column equilibrated with buffer B containing 150 mM NaCl. The active fractions were recovered in the flow-through fractions and loaded onto a hydroxylapatite (4 ml) column equilibrated with buffer C (30 mM potassium phosphate, pH 7.0, 12.5 mM β-glycerophosphate, 0.1 mM EGTA, 1 mM PMSF, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate). After washing the column, proteins were eluted with a 60 ml linear 30−200 mM potassium phosphate gradient. The active fractions (eluting at 100−160 mM potassium phosphate) were pooled and dialysed against buffer D (20 mM MES, pH 6.5, 12.5 mM β-glycerophosphate, 1 mM EGTA, 1 mM PMSF, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate). The dialysate was loaded onto an FPLC Mono-S Pharmacia HR 5/5 column equilibrated with buffer D. After washing the column, proteins were eluted with a 15 ml linear 0−400 mM NaCl gradient. The active fractions (eluting at 160−190 mM NaCl) were stored at −20°C as 50% glycerol solutions.

MAP kinase activator assays

To detect the ability to activate the MBP kinase activity of recombinant MAP kinase, samples for assay were incubated for 20 min at 30°C with 10 μg/ml purified recombinant MAP kinase and 50 μM [γ-^{32}P]ATP (1 μCi) in 20 mM Tris−Cl, pH 7.5, 15 mM $MgCl_2$, 2 mM EGTA and 150 μg/ml MBP in a final volume of 24 μl. To detect the ability to phosphorylate recombinant MAP kinase, MBP was omitted in the above incubation. The reaction was stopped by the addition of Laemmli's sample buffer and boiling. After electrophoresis, bands were detected by autoradiography. For quantification, an image analyser (FUJIX BAS 2000) was used. Alternatively, samples were incubated with recombinant MAP kinase and nonlabelled ATP and subsequently subjected to the kinase detection assay within gels containing MBP (Gotoh *et al.*, 1990b). In this assay, the kinase activity of activated recombinant MAP kinase was detected by means of a kinase reaction in a polyacrylamide gel containing 0.5 mg/ml MBP following denaturation and renaturation after SDS−PAGE of the sample. For assaying the activator activity in *Xenopus* extracts (Figures 8 and 9), extracts (0.2 ml) were loaded onto a DEAE−cellulose column (0.5 ml) and the activator activity of the flow-through fraction was determined by the kinase detection assay within MBP-containing gels, after incubation with recombinant MAP kinase and ATP as above. For quantification, the radioactivity of the gel corresponding to the electrophoretic position of the activated recombinant MAP kinase was counted.

Phosphatase treatment

Samples for assay were dialysed against a buffer solution (20 mM Tris−Cl, pH 7.0, 2 mM EGTA, 110 mM NaCl, 2 mM dithiothreitol) to exclude phosphatase inhibitors. The samples were then incubated for 0−30 min at 30°C with the catalytic subunit of protein phosphatase 2A (1 μg/ml). For termination of the phosphatase reaction, okadaic acid was added to a final concentration of 10 μM and immediately assayed for the activator activity.

Oocyte labelling and purification of labelled MAP kinase activator

Mature oocytes were labelled with 0.3 mCi/ml [^{32}P]orthophosphate in phosphate free modified OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 35 mM HEPES, pH 7.8) for 3 h and then extracts were prepared and mixed with one-third volume of DEAE−cellulose resin. After 20 min incubation at 0°C, the unbound fraction was collected. In separate experiments, extracts obtained from 25 ml dejellied mature oocytes without labelling were passed through a DEAE−cellulose column (15 ml) and the non-labelled flow-through fraction was obtained. These two fractions were mixed and then loaded onto a heparin−Sepharose (4 ml) column

equilibrated with buffer B. From this step, the purification procedure for MAP kinase activator was performed as described above on a ¼ scale except for the use of a phosphocellulose (1 ml) column instead of a final FPLC Mono-S column.

Immunoblotting

After SDS−PAGE, proteins were transferred to Immobilon (Millipore) membrane in a solution containing 25 mM Tris, 192 mM glycine and 20% methanol. After blocking with 1% BSA in TBS (500 mM NaCl, 20 mM Tris−Cl, pH 7.5), membranes were incubated with the anti-MAP kinase antibody (DME or QET) at a dilution of 1:1000 or with anti-phosphotyrosine antibody (PY20, ICN) at a dilution of 1:500. Reacted proteins were detected by HRP-conjugated anti-rabbit (or mouse) IgG antibody.

Purification of native inactive MAP kinase

Extracts were prepared from immature oocytes (10 ml packed volume) as described previously (Gotoh *et al.*, 1991a). Inactive MAP kinase, detected by immunoblotting with anti-MAP kinase antibody (DME), was purified by sequential chromatography on DEAE−cellulose (eluting at 0.1−0.4 M NaCl), phenyl−Sepharose (eluting at 40−70% ethylene glycol), polylysine−agarose (eluting at 0.16−0.52 M NaCl), hydroxylapatite (eluting at 0.19−0.32 M potassium phosphate) and heparin−Sepharose (eluting at 0.21−0.36 M NaCl), using buffer conditions described previously (Gotoh *et al.*, 1990b).

Microinjection

Microinjection of purified *Xenopus* MAP kinase activator or purified MPF into immature oocytes was carried out in modified Barth's medium at 21°C with an IM-1 microinjection apparatus (Narishige, Tokyo) as described (Gotoh *et al.*, 1991b). *Xenopus* MPF was purified from mature oocytes by $p13^{suc1}$ affinity chromatography as described by Labbé *et al.* (1989).

Preparation of Xenopus oocyte extracts

Immature *Xenopus* oocytes (stage VI) were prepared as described previously (Gotoh *et al.*, 1991a). Maturation was induced by treatment with 10 μM progesterone (Sigma). Unfertilized eggs were obtained by injecting 400 IU chorionic gonadotropin into the dorsal lymph sac of females. Approximately 10−12 h later, eggs were squeezed from induced females directly into MMR buffer (0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA and 5 mM HEPES, pH 7.8). Interphase eggs were obtained 60 min after fertilization and the extracts were prepared as described previously (Gotoh *et al.*, 1991b).

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References

Ahn,N.G. and Krebs,E.G. (1990) *J. Biol. Chem.*, **265**, 11495−11501.
Ahn,N.G., Weiel,J.E., Chan,C.P. and Krebs,E.G. (1990) *J. Biol. Chem.*, **265**, 11487−11494.
Ahn,N.G., Seger,R., Bratlien,R.L., Diltz,C.D., Tonks,N.K. and Krebs,E.G. (1991) *J. Biol. Chem.*, **266**, 4220−4227.
Anderson,N.G., Maller,J.L., Tonks,N.K. and Sturgill,T.W. (1990) *Nature*, **343**, 651−653.
Boulton,T.G., Yancopoulos,G.D., Gregory,J.S., Slaughter,C., Moomaw,C., Hsu,J. and Cobb,M.H. (1990) *Science*, **249**, 64−67.
Boulton,T.G. and Cobb,M.H. (1991) *Cell Regulation*, **2**, 357−371.
Boulton,T.G. *et al.* (1991) *Cell*, **65**, 663−675.
Chung,J., Pelech,S.L. and Blenis,J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4981−4985.
Ely,C.M., Oddie,K.M., Litz,J.S., Rossomando,A.J., Kanner,S.B., Sturgill,T.W. and Parsons,S.J. (1990) *J. Cell Biol.*, **110**, 731−742.
Ferrell,J.E., Wu,M., Gerhart,J.C. and Martin,G.S. (1991) *Mol. Cell. Biol.*, **11**, 1965−1971.
Gomez,N., Tonks,N.K., Morrison,C., Harmer,T. and Cohen,P. (1990) *FEBS Lett.*, **271**, 119−122.
Gomez,N. and Cohen,P. (1991) *Nature*, **353**, 170−173.
Gotoh,Y., Nishida,E. and Sakai,H. (1990a) *Eur. J. Biochem.*, **193**, 671−674.
Gotoh,Y., Nishida,E., Yamashita,T., Hoshi,M., Kawakami,M. and Sakai,H. (1990b) *Eur. J. Biochem.*, **193**, 661−669.
Gotoh,Y., Nishida,E., Matsuda,S., Shiina,N., Kosako,H., Shiokawa,K., Akiyama,T., Ohta,K. and Sakai,H. (1991a) *Nature*, **349**, 251−254.
Gotoh,Y. *et al.* (1991b) *EMBO J.*, **10**, 2661−2668.
Gregory,J.S., Boulton,T.G., Sang,B.-C. and Cobb,M.H. (1989) *J. Biol. Chem.*, **264**, 18397−18401.
Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42−52.
Haystead,T.A.J., Weiel,J.E., Litchfield,D.W., Tsukitani,Y., Fischer,E.H. and Krebs,E.G. (1990) *J. Biol. Chem.*, **265**, 16571−16580.
Hoshi,M., Nishida,E. and Sakai,H. (1988) *J. Biol. Chem.*, **263**, 5396−5401.
Hoshi,M., Nishida,E. and Sakai,H. (1989) *Eur. J. Biochem.*, **184**, 477−486.
Labbé,J.-C., Capony,J.-P., Caput,D., Cavadore,J.-C., Derancourt,J., Kaghad,M., Lelias,J.-M., Picard,A. and Dorée,M. (1989) *EMBO J.*, **8**, 3053−3058.
Maller,J. (1990) *Biochemistry*, **29**, 3157−3166.
Miyasaka,T., Chao,M., Sherline,P. and Saltiel,A. (1990) *J. Biol. Chem.*, **265**, 4730−4735.
Murray,A.W. and Kirschner,M.W. (1989) *Science*, **246**, 614−621.
Nel,A., Hanekom,C., Rheeder,A., Williams,K., Pollack,S., Katz,R. and Landreth,G. (1990) *J. Immunol.*, **144**, 2683−2689.
Nurse,P. (1990) *Nature*, **344**, 503−508.
Payne,D.M., Rossomando,A., Martino,P., Erickson,A.K., Her,J.-H., Shabanowitz,J., Hunt,D.F., Weber,M.J. and Sturgill,T.W. (1991) *EMBO J.*, **10**, 885−892.
Pelech,S.L., Tombes,R.M., Meijer,L. and Krebs,E.G. (1988) *Dev. Biol.*, **130**, 28−36.
Posada,J., Sanghera,J., Pelech,S., Aebersold,R. and Cooper,J. (1991) *Mol. Cell. Biol.*, **11**, 2517−2528.
Ray,L.B. and Sturgill,T.W. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1502−1506.
Ray,L.B. and Sturgill,T.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3753−3757.
Rossomando,A., Payne,D.M., Weber,M. and Sturgill,T.W. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6940−6943.
Sanghera,J., Paddon,H., Bader,S. and Pelech,S. (1990) *J. Biol. Chem.*, **265**, 52−57.
Seger,R. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 6142−6146.
Sturgill,T.W., Ray,L.B., Erikson,E. and Maller,J.L. (1988) *Nature*, **334**, 715−718.
Tsao,H., Aletta,J.M. and Greene,L.A. (1990) *J. Biol. Chem.*, **265**, 15471−15480.

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