The EMBO Journal vol.10 no.9 pp.2661 - 2668, 1991

Xenopus M phase MAP kinase: isolation of its cDNA and activation by MPF

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Communicated by P.Nurse

MAP kinase is activated and phosphorylated during M phase of the *Xenopus* oocyte cell cycle, and induces the interphase–M phase transition of microtubule dynamics *in vitro*. We have carried out molecular cloning of *Xenopus* M phase MAP kinase and report its entire amino acid sequence. There is no marked change in the MAP kinase mRNA level during the cell cycle. Moreover, studies with an anti-MAP kinase antiserum indicate that MAP kinase activity may be regulated post-translationally, most likely by phosphorylation. We show that MAP kinase can be activated by microinjection of MPF into immature oocytes or by adding MPF to cell-free extracts of interphase eggs. These results suggest that MAP kinase functions as an intermediate between MPF and the interphase–M phase transition of microtubule organization.

Key words: kinase cascade/MAP kinase/MPF/M phase

Introduction

A burst in kinase activation and protein phosphorylation (Maller *et al.*, 1977; Karsenti *et al.*, 1987; Cicirelli *et al.*, 1988; Pelech *et al.*, 1988; Sanghera *et al.*, 1990) is important in triggering the dramatic reorganization of the cell including chromosome condensation, cytoskeletal reorganization and nuclear envelope breakdown during the transition from interphase to mitosis. M phase promoting factor (MPF), a $p34^{cdc2}$–cyclin complex (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1988, 1989; Lohka *et al.*, 1988; Meijer *et al.*, 1989; Gautier *et al.*, 1990) that is a central control element for the onset of M phase (Kishimoto, 1988; Murray and Kirschner, 1989; Maller, 1990; Nurse, 1990), has recently been shown to phosphorylate directly lamin and vimentin, resulting in the dissolution of lamina and the disassembly of vimentin intermediate filaments, respectively (Chou *et al.*, 1990; Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). It is not clear, however, to what degree MPF acts directly on the crucial protein substrates involved in major M phase events. Lewin (1990) proposed two, not mutually exclusive, possibilities. First, MPF may function as a 'workhorse' that directly phosphorylates the crucial substrates to execute cell reorganization. Second, MPF may function as a 'master regulator' that elicits its effects by way of downstream kinases.

Mammalian MAP kinase is a serine/threonine kinase whose activation and phosphorylation on tyrosine and threonine residues are rapidly induced by a variety of mitogens (Ray and Sturgill, 1987, 1988a,b; Hoshi *et al.*, 1988, 1989; Rossomando *et al.*, 1989; Gotoh *et al.*, 1990a,b) and which is considered to have a critical role in a network of protein kinases in mitogenic signal transduction (Sturgill *et al.*, 1988; Anderson *et al.*, 1990; Maller, 1990). We have shown that a *Xenopus* 42 kDa MAP kinase, closely related to mammalian MAP kinase, is activated and phosphorylated on tyrosine and serine residues during M phase of *Xenopus* oocyte cell cycles and that the interphase–M phase transition of microtubule dynamics can be induced by the addition of purified *Xenopus* M phase MAP kinase to interphase extracts (Gotoh *et al.*, 1991). Thus, MAP kinase may function at M phase as well as at G_0/G_1 phase. Although it is evident that MAP kinase activation at G_0/G_1 phase is triggered by extracellular stimuli such as peptide growth factors, it is unknown what triggers the activation of MAP kinase during M phase. A straightforward idea is that MPF acts as an upstream activator of MAP kinase. But, this should be closely examined because it is likely that there must exist M phase kinases that are not downstream of MPF. For example, previous studies with *Schizosaccharomyces pombe* have revealed at least two protein kinases upstream of $p34^{cdc2}$ (Russell and Nurse, 1987a,b), and Solomon *et al.* (1990) have shown tyrosine and threonine phosphorylations of $p34^{cdc2}$ just prior to the dephosphorylation-induced kinase activation and another threonine phosphorylation in the active form of $p34^{cdc2}$ (see also Lewin, 1990).

In this study, we demonstrate that MAP kinase can be activated by microinjection of MPF into immature *Xenopus* oocytes, and further demonstrate an *in vitro* system, where the addition of MPF to cell-free extracts of interphase eggs can induce MAP kinase activation via phosphorylation reactions. In addition, the molecular cloning and sequencing of a *Xenopus* M phase MAP kinase cDNA described in this report has revealed the whole amino acid sequence of MAP kinase. Northern blotting analyses have shown that there is no marked change in MAP kinase mRNA level during the cell cycle. Western blotting and immunoprecipitation experiments with a rabbit antiserum to the near C-terminal, 14 amino acid sequence that is conserved between mammalian and amphibian MAP kinases indicate that the MAP kinase activity is regulated post-translationally, most likely by phosphorylation, in *Xenopus* oocytes. These results demonstrate convincingly that MAP kinase is activated during M phase under the control of MPF and may constitute part of kinase cascades downstream from MPF, and

therefore suggest that MAP kinase acts as an intermediate between MPF and M phase cellular events.

Results

Molecular cloning of Xenopus M phase MAP kinase

We previously reported purification and characterization of *Xenopus* M phase-activated MAP kinase with a molecular mass of 42 kDa and showed that it is biochemically and enzymatically related to mammalian mitogen-activated MAP kinase (Gotoh *et al.*, 1991). To elucidate the structure of the *Xenopus* kinase, we isolated its cDNA clones. A *Xenopus* oocyte cDNA library was screened with two oligonucleotide probes which were synthesized according to partial amino acid sequences of lysylendopeptidase-digested fragments of purified kinase. A clone, positive to both probes, that was sequenced contained an open reading frame which encodes a protein consisting of 361 amino acid residues with a calculated molecular mass of 41.3 kDa (Figure 1). The predicted amino acid sequence contains all the conserved residues and 11 subdomains of protein kinases and shows characteristics of serine/threonine kinases (Hanks *et al.*, 1988). Figure 2 shows that this *Xenopus* kinase is 84% identical to rat insulin-stimulated MAP kinase (= ERK1) whose partial sequence was recently reported (Boulton *et al.*, 1990). The extensive sequence similarity between both kinases together with our previous biochemical comparison (Gotoh *et al.*, 1990b, 1991) shows conclusively that *Xenopus* M phase-activated MAP kinase is a homolog of mammalian mitogen-activated MAP kinase, and may imply the significance of MAP kinase function in fundamental biological activities. While this paper was in review, Posada *et al.*

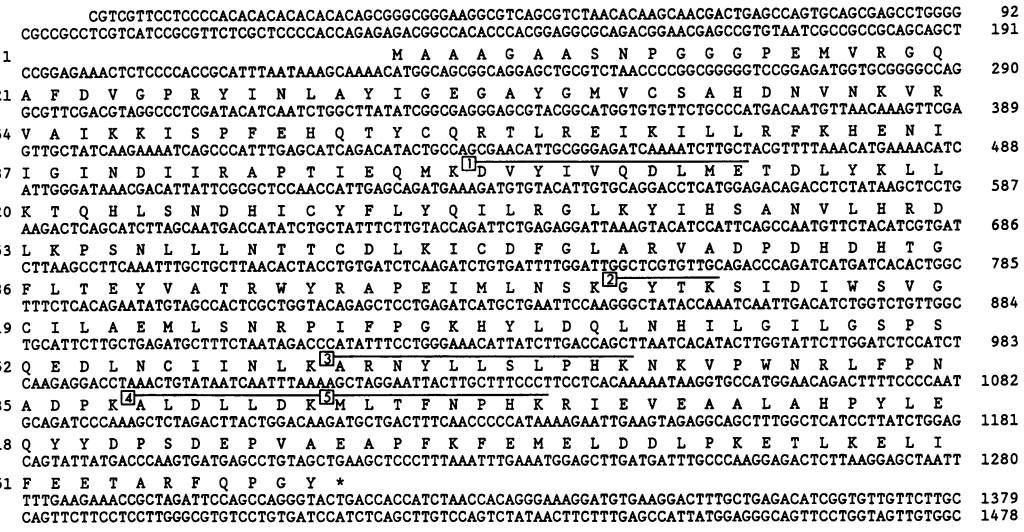

Fig. 1. Nucleotide sequence of the *Xenopus* M phase-activated MAP kinase cDNA and its predicted amino acid sequence. Nucleotides and amino acids are numbered on the right and left, respectively. Peptide sequences derived from purified kinase are overlined and numbered in squares. The position of translational initiator ATG is likely to be true because of the consensus sequence for eukaryotic initiator sites (AAACATGG) (Kozak, 1987), the predicted molecular mass (41 256 Daltons) and the termination codon 165 bp upstream of it. The N-terminal region (~15 amino acids) of *Xenopus* MAP kinase as well as ERK1 (Boulton *et al.*, 1990) is rich in non-polar, hydrophobic amino acids. This might explain the high affinity of MAP kinase for hydrophobic matrices such as phenyl-Sepharose.

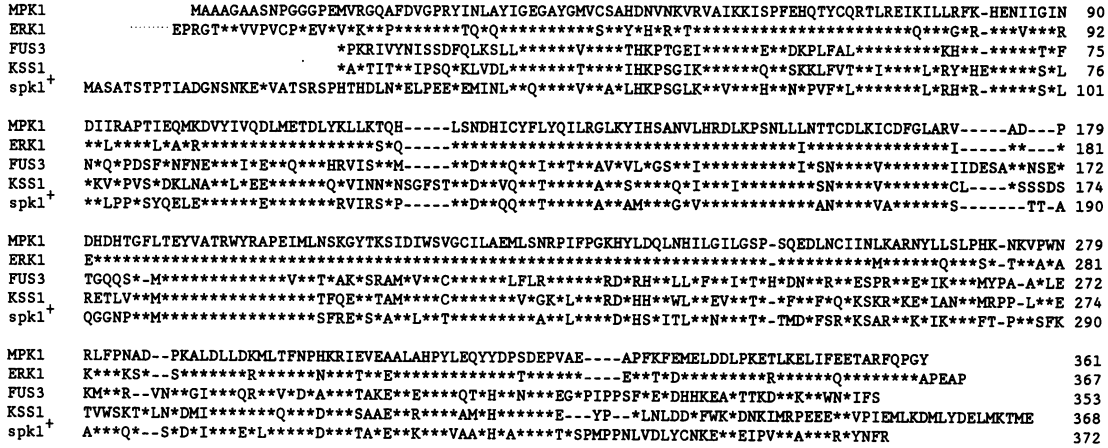

Fig. 2. Comparison of *Xenopus* M phase MAP kinase with other known kinases. MPK1, *Xenopus* M phase-activated MAP kinase; ERK1 (Boulton *et al.*, 1990), rat insulin-stimulated MAP kinase; FUS3 (Elion *et al.*, 1990) and KSS1 (Courchesne *et al.*, 1989), *S.cerevisiae* serine/threonine kinases mediating the yeast rseponse to pheromones; spk1$^+$ (Toda *et al.*, 1991), an *S.pombe* protein kinase conferring staurosporine resistance when carried on multicopy plasmids. Asterisks indicate identical residues with those of MPK1. Dashes indicate spaces introduced to maximally align sequences. MPK1 is 84%, 50%, 51% and 52% identical to ERK1, FUS3, KSS1 and spk1$^+$, respectively, over their shared lengths.

(1991) also reported a *Xenopus* MAP kinase sequence that was slightly different from our sequence. This difference might reflect allelic variation or different genes.

The striking similarity exists (Figure 2) between MAP kinase and two *Saccharomyces cerevisiae* protein kinases (FUS3 and KSS1) mediating the yeast response to pheromones (Courchesne *et al.*, 1989; Elion *et al.*, 1990), as previously pointed out by Cobb and co-workers (Boulton *et al.*, 1990). In addition, *Xenopus* MAP kinase is 52% identical to an *S.pombe* protein kinase spk1$^+$ over their shared lengths (Figure 2). The spk1$^+$ confers staurosporine resistance when carried on multicopy plasmids and exists upstream of *S.pombe* AP-1 (= pap1$^+$) (Toda *et al.*, 1991). These may suggest evolutionarily conserved functions for these kinases.

The MAP kinase activity may be regulated post-translationally

A 3.3 kb MAP kinase transcript was expressed in all the *Xenopus* tissues examined, with the highest concentration in brain (Figure 3A). To investigate whether or not the expression level of MAP kinase is correlated with the activation of MAP kinase activity during M phase, Northern blot analysis was carried out. There was no marked change in the level of MAP kinase mRNA during the meiotic (Figure 3B) and mitotic (data not shown) cell cycles whereas the activity of MAP kinase oscillated (Figure 3B).

We produced anti-MAP kinase antiserum by immunizing a rabbit with a synthetic peptide DMELDDLPKERLKE, the near C-terminal 14 amino acid sequence of ERK1 that is highly conserved among ERK1 and *Xenopus* MAP kinase (see Figures 1 and 2). This antiserum reacted strongly with purified rat EGF-activated MAP kinase [Figure 4A, lane 1, ~43 kDa and ~41 kDa, both polypeptides have been shown to possess the kinase activity in our previous studies (Gotoh *et al.*, 1990a,b)]. This antiserum reacted with purified *Xenopus* MAP kinase, too (~42 kDa, Figure 4A, lane 2) and the 42 kDa polypeptide that was immunoprecipitated with the antiserum from *Xenopus* mature oocyte extracts possessed the kinase activity (lane 3). The Western blotting with this antiserum further showed that the amount of MAP kinase protein did not change markedly during *Xenopus* oocyte maturation and that the active *Xenopus* MAP kinase at maturation migrated more slowly in electrophoresis than the inactive form of kinase in immature oocytes did (Figure 4B, lanes 1 and 2). This electrophoretic mobility shift may result from phosphorylation, since only the electrophoretically retarded band possessed the kinase activity (Figure 4B, lanes 3 and 4) and immunoprecipitation from ^{32}P-labeled oocytes demonstrated almost no labeling of the inactive MAP kinase and strong labeling of the active kinase (Figure 4B, lanes 5 and 6). The immunoprecipitated active M phase MAP kinase contained phosphotyrosine (data

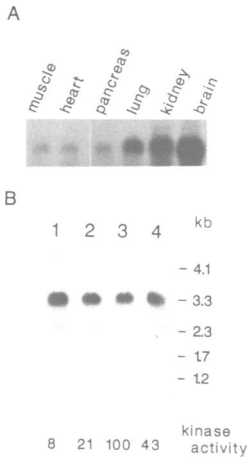

Fig. 3. Northern blotting analysis. (**A**) Distribution of MAP kinase mRNA in *Xenopus* tissues. Total RNA (10 μg each) from skeletal muscle, heart, pancreas, lung, kidney and brain was analyzed with a probe of 0.6 kb fragment of *Xenopus* M phase MAP kinase cDNA. A major 3.3 kb MAP kinase transcript was detected in all tissues examined. (**B**) Analysis of MAP kinase mRNA during *Xenopus* oocyte maturation. Immature *Xenopus* oocytes (stage VI) were treated with 10 μM progesterone for 0 h (lane 1), 3 h (lane 2), 6 h (lane 3) and 9 h (lane 4). Northern blotting was carried out as in (A) and the MAP kinase activity of extracts obtained from oocytes (shown in arbitrary units below each lane) was determined. Positions of glyoxylated DNA standards are indicated.

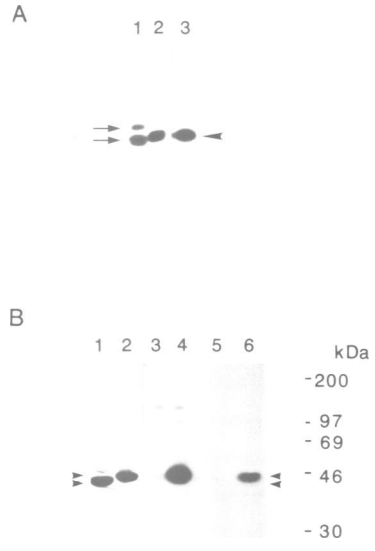

Fig. 4. Analyses with the anti-MAP kinase antiserum. (**A**) The reactivity of the anti-DMELDDLPKERLKE serum. The antiserum against the 14 amino acid synthetic peptide (see text) was prepared as described in Materials and methods. Rat EGF-activated MAP kinase purified from 3Y1 cells (Gotoh *et al.*, 1990b) was subjected to immunoblotting with this antiserum (lane 1). Arrows indicate two closely-related mammalian MAP kinases (Gotoh *et al.*, 1990b). *Xenopus* M phase MAP kinase purified as described (Gotoh *et al.*, 1991) was analyzed by immunoblotting with this antiserum (lane 2). An extract of *Xenopus* progesterone-treated oocytes was subjected to immunoprecipitation with this antiserum. The precipitate was electrophoresed in an MBP-containing SDS−polyacrylamide gel, subjected to denaturation and renaturation, and then underwent the kinase detection assay within the gel (lane 3). An arrowhead indicates the position of purified *Xenopus* MAP kinase. (**B**) Post-translational activation of *Xenopus* MAP kinase by progesterone. *Xenopus* extracts were electrophoresed, transferred and immunoblotted with the antiserum (lanes 1 and 2). *Xenopus* extracts were electrophoresed in an MBP-containing polyacrylamide gel and assayed for the kinase activity within the gel (lanes 3 and 4). Lanes 1 and 3, immature oocytes (stage VI); lanes 2 and 4, immature oocytes treated with progesterone for 6 h. Immature *Xenopus* oocytes prelabeled with [^{32}P]orthophosphate were incubated in the absence (lane 5) or presence (lane 6) of 10 μM progesterone for 6 h. Extracts prepared from these oocytes were immunoprecipitated with the antiserum and analyzed by SDS−PAGE and autoradiography.

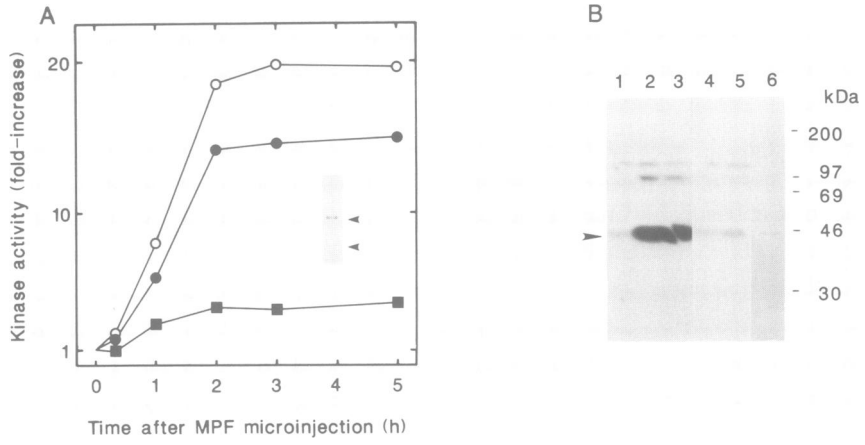

Fig. 5. Activation of MAP kinase by microinjection of MPF into immature *Xenopus* oocytes. Purified MPF prepared from starfish oocytes [a silver-stained SDS−polyacrylamide gel is shown in inset of (**A**); upper arrowhead, cyclin; lower arrowhead, $p34^{cdc2}$] was microinjected into immature *Xenopus* oocytes (stage VI). Various times after microinjection the oocytes were homogenized, and kinase activities in the extracts toward MAP2 (●, 20 μg/ml), MBP (○, 1 mg/ml) and histone H1 (■, 1 mg/ml) were determined (A). Kinase activities at zero time were 0.86, 2.4 and 1.4 fmol/min/μl of extract for MAP2, MBP and H1, respectively. The amount of MPF being microinjected into the oocyte corresponded roughly to 8% of that in the mature (metaphase II) oocyte, as determined by measuring the histone H1 kinase activity. The germinal vesicle breakdown occurred in microinjected oocytes within 2 h. The kinase activities toward histone H1, MAP2 and MBP increased ~4-, 15- and 20-fold, respectively, 2−5 h after MPF microinjection. In (**B**), the kinase activity was detected by kinase reaction in a polyacrylamide gel containing 0.5 mg/ml MBP following denaturation and renaturation after SDS−PAGE of the extracts. Times after microinjection of MPF were 0 (lane 1), 2 h (lane 2) and 3 h (lane 3). Lane 4, 3 h after the buffer solution alone was injected; lane 5, incubation of the oocytes with 10 μM progesterone for 2 h, when germinal vesicle breakdown did not occur. A silver-stained polyacrylamide gel of purified *Xenopus* M phase-activated MAP kinase is shown (lane 6).

not shown), as is consistent with our previous data (Gotoh *et al.*, 1991). These results, taken together, suggest that the activity of MAP kinase may be regulated post-translationally.

Activation of MAP kinase by microinjection of MPF into immature Xenopus oocytes

It is possible that MAP kinase activation is triggered by MPF (see Introduction), as we observed that activation of MAP kinase at M phase in *Xenopus* oocytes lags slightly behind activation of the histone H1 kinase activity (Gotoh *et al.*, 1991), a measure of MPF or $p34^{cdc2}$ kinase. To examine this possibility, starfish MPF purified by $p13^{scu1}$ affinity chromatography was microinjected into *Xenopus* immature oocytes. MAP kinase activity in oocyte extracts, assayed by utilizing microtubule-associated protein 2 (MAP2), or myelin basic protein (MBP) as exogenous substrates, increased 20 min after microinjection of MPF and attained the apparent steady-state level after 2 h (Figure 5A, ○ and ●). The detection of the kinase activity in polyacrylamide gels containing MBP after SDS−PAGE of the oocyte extracts confirmed that the 42 kDa *Xenopus* MAP kinase was prominently activated (Figure 5B, lanes 1, 2 and 3). Microinjection of the buffer solution alone did not induce activation of MAP kinase (lane 4). On the other hand, microinjection of purified *Xenopus* MAP kinase did not increase the histone H1 kinase activity in immature oocytes (data not shown). These results suggest that MPF acts as an upstream activator of MAP kinase.

In vitro activation of MAP kinase by MPF in cell-free extracts

To study the mechanism of MAP kinase activation by MPF, it is desirable to establish an *in vitro* system. We used cell-free extracts prepared from *Xenopus* interphase eggs. The addition of purified MPF into interphase extracts of activated mature eggs resulted in a time-dependent increase in MAP2/MBP kinase activity (Figure 6A, —○—). The increase in the kinase activity reflected MAP kinase activation, as revealed by the kinase assays within MBP-containing polyacrylamide gels after SDS−PAGE (Figure 6B). No activation of the histone H1 kinase activity occurred when purified *Xenopus* MAP kinase was added to the interphase extracts (Figure 6A, --■--). Thus, in this *in vitro* system also, MPF can act as an activator of MAP kinase.

To determine whether the kinase activity of MPF is essential for triggering MAP kinase activation, we added staurosporine with MPF to the interphase extracts, as staurosporine at μM level was found to inhibit the histone H1 kinase activity of purified MPF under our assay conditions (Figure 7A, open circles). Figure 7 (A and B) shows clearly that staurosporine inhibited the activation of MAP kinase by MPF in a dose-dependent manner. This staurosporine concentration dependence (Figure 7A, closed bars) was almost the same as that for the inhibition of the HI kinase activity of MPF (Figure 7A, open circles). Therefore, the MPF-induced activation of MAP kinase may be mediated by MPF-catalyzed phosphorylation events.

We have previously shown that purified *Xenopus* M phase MAP kinase is inactivated by treatment with protein phosphatase 2A (Gotoh *et al.*, 1991). To test the possibility that MPF can directly phosphorylate and activate MAP kinase, the purified *Xenopus* MAP kinase previously inactivated by protein phosphatase 2A was incubated with purified MPF. Neither reactivation nor phosphorylation of the MAP kinase could be detected whereas cyclin was heavily phosphorylated (data not shown). This is consistent with the fact that there exists no consensus sequence for phosphorylation by MPF (Suzuki, 1989; Maller, 1990) in *Xenopus* MAP kinase sequence (see Figure 1).

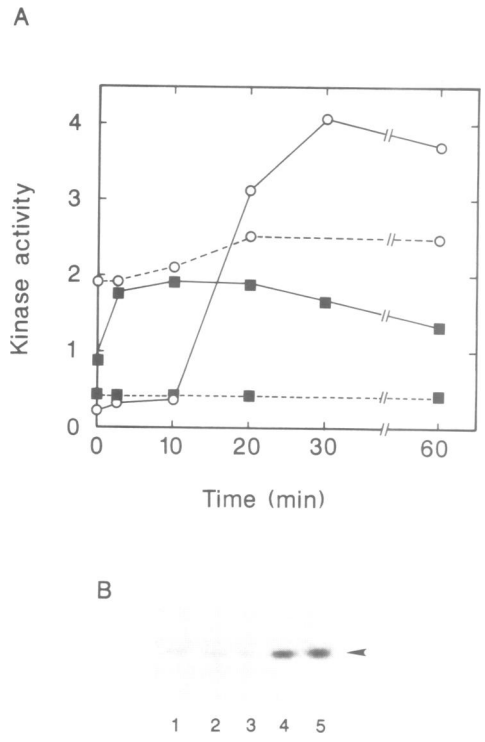

Fig. 6. Activation of MAP kinase by MPF in cell-free extracts. (**A**) Purified MPF (solid lines, ○ ■) or purified *Xenopus* M phase MAP kinase (broken lines, ○ ■) was added to interphase extracts of *Xenopus* activated eggs and various times after incubation at 22°C the extracts were diluted 25-fold and assayed for the kinase activity toward MBP (○) and histone H1 (■). The amounts of MPF and MAP kinase being added to the extracts corresponded to 26% and 65%, respectively, of those in metaphase II extracts, as determined by measuring histone H1 kinase and MAP kinase activities. The time courses of changes in the kinase activity are shown. One unit indicates 0.31 and 0.055 pmol/min/μl of extract for MBP and H1, respectively. In (**B**), the kinase activity was detected in polyacrylamide gels containing MBP after SDS−PAGE. The extracts being incubated with MPF were analyzed. Times for incubation with MPF were 0 (lane 1), 2.5 (lane 2), 10 (lane 3), 20 (lane 4) and 30 min (lane 5). The electrophoretic position of active *Xenopus* MAP kinase is indicated by an arrow. When MPF was added to metaphase II extracts, ~1.3-fold activation of MAP kinase activity occurred (data not shown).

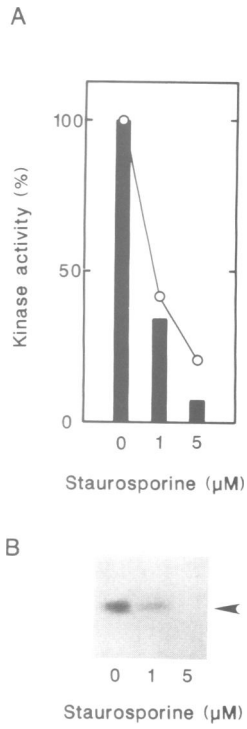

Fig. 7. Inhibition of MPF-induced MAP kinase activation by staurosporine. Purified MPF was added to the interphase extracts in the presence of indicated concentrations of staurosporine. Thirty minutes after the MPF addition the MAP kinase activity of the extracts was determined by utilizing MBP as an exogenous substrate after 25-fold dilution of the extracts [filled columns, (**A**)] or in polyacrylamide gels containing MBP after SDS−PAGE (**B**). The electrophoretic position of active *Xenopus* MAP kinase is indicated by an arrow. Open circles [○ in (A)] show the kinase activity of purified MPF toward H1 in the absence of the extracts. Kinase activity in the absence of staurosporine is regarded as 100%. The effect of staurosporine on MAP kinase activity is negligible after dilution (<3% inhibition at 0.2 μM staurosporine) or in the kinase detection assay in polyacrylamide gels.

Discussion

The present study on the molecular cloning and sequencing of a *Xenopus* M phase 42 kDa serine/threonine kinase has demonstrated that it is 84% identical to mammalian mitogen-activated MAP kinase whose partial sequence was previously reported (Boulton *et al.*, 1990). Both kinases are very similar not only in their structural (Figure 2) and immunological (Figure 4) properties but also in their enzymatic and biochemical properties (Gotoh *et al.*, 1991), suggesting that these kinases belong to the same family of serine/threonine kinase, collectively termed as MAP kinase. The existence of related kinases in yeasts (see Results) may suggest universality and importance of this family of protein kinases.

Previously, we showed the activation of *Xenopus* MAP kinase during M phase of oocyte cell cycles (Gotoh *et al.*, 1991), but it has been unknown whether the activation of M phase MAP kinase is regulated by MPF. Experiments with anti-MAP kinase antiserum which was produced based on the sequence data of *Xenopus* and rat MAP kinase indicate that the activity of MAP kinase is regulated post-translationally. Furthermore, we have clearly shown that *Xenopus* MAP kinase can be activated by microinjection of MPF into immature oocytes or by adding MPF to interphase extracts obtained from activated mature eggs, demonstrating that MPF can act as an upstream activator of MAP kinase. This *in vitro* system provides the important implications concerning regulation of M phase cellular events by MPF. Karsenti and co-workers reported, using the same or similar *in vitro* system, that microtubules nucleated by the added mammalian centrosomes in interphase extracts grow faster and are longer at steady-state than those in metaphase II extracts and that the interphase−M phase transition of microtubule arrays is under the control of MPF or $p34^{cdc2}$ kinase (Verde *et al.*, 1990). Microtubules formed in M phase extracts are more dynamic and change into the shrinking phase more frequently than those in interphase extracts (Belmont *et al.*, 1990). We previously showed that the interphase−M phase transition of microtubule dynamics can be induced by the addition of purified *Xenopus* M phase MAP kinase to interphase extracts (Gotoh *et al.*, 1991). In this *in vitro* cell-free system, MAP kinase can be activated by the addition of MPF, while no activation of $p34^{cdc2}$

kinase or MPF occurs after the addition of MAP kinase (Figure 6). Therefore, the effect of MPF or $p34^{cdc2}$ on microtubule dynamics in interphase extracts may be mediated, at least partly, by activation of MAP kinase. Thus, M phase MAP kinase functions as an intermediate between MPF and the interphase−M phase transition of microtubule organization (Figure 8). In other words there exists the kinase cascade, that controls M phase events, downstream of MPF. It is, of course, possible that MPF/$p34^{cdc2}$ plays a direct role in the change in microtubule arrays by phosphorylating regulatory proteins involved in microtubule organization (Figure 8). The elucidation of the direct substrates of MAP kinase and MPF in this system will clarify roles of these two M phase-activated kinases in one of the major M phase cellular events, microtubule reorganization.

S6 kinase II has also been shown to be activated by microinjection of MPF into oocytes (Erikson and Maller, 1989; Maller, 1990), and the mammalian adipocyte MAP kinase can phosphorylate and activate the previously inactivated S6 kinase II *in vitro* (Sturgill *et al.*, 1988). These data together with the present result suggest the existence of the kinase cascade of MPF → MAP kinase → S6 kinase (Figure 8). A recent report has shown, however, that there are at least two distinct pathways for S6 kinase activation, as mammalian 70 K S6 kinase may not be downstream of MAP kinase (Ballou *et al.*, 1991).

The mechanism by which MPF activates MAP kinase remains to be elucidated. As a kinase inhibitor staurosporine inhibits the MPF-induced activation of MAP kinase in the same dose-dependent manner as it inhibits the histone H1 kinase activity of MPF, it may be certain that MPF-catalyzed phosphorylation reactions are required for the MAP kinase activation. Since staurosporine may inhibit the activity of a wide variety of protein kinases although it is best known as a protein kinase C inhibitor, it is also possible that staurosporine inhibits the intermediate kinases between MPF and MAP kinase (see below). Under our assay conditions MPF cannot directly phosphorylate MAP kinase previously inactivated by phosphatase 2A treatment. However it is still possible that MPF is responsible for direct phosphorylation of MAP kinase *in vivo*, as our *in vitro* assay system might lack some essential conditions required for physiological kinase reaction of MPF. *Xenopus* MAP kinase is presumed to be activated by its phosphorylation on tyrosine and serine residues (Gotoh *et al.*, 1991; this paper). Then, the simplest assumption is that two protein kinases, a tyrosine kinase and a serine/threonine kinase, existing downstream of MPF, phosphorylate MAP kinase. It should be noted that a factor activating MAP kinase is not necessarily a protein kinase and can be an inducer of MAP kinase autophosphorylation. In any event, identification of the MAP kinase activator and elucidation of its relationship to MPF must be required for full understanding of the activation mechanism of MAP kinase. The molecular cloning and sequencing of MAP kinase as well as the *in vitro* activation system described in this study may provide crucial bases for these analyses.

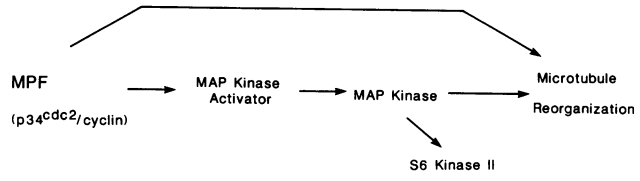

Fig. 8. A simple model illustrating the role of MAP kinase at M phase. MAP kinase activation at M phase is under the control of MPF (this study) and the activated MAP kinase plays a critical role in inducing the interphase−M phase transition of microtubule organization (Gotoh *et al.*, 1991). MPF itself may also affect the microtubule arrays (Verde *et al.*, 1990). MAP kinase has previously been shown to activate *Xenopus* S6 kinase II *in vitro* (Sturgill *et al.*, 1988). Thus, there exist kinase cascades that control M phase events, where MAP kinase functions downstream of MPF. See text for details.

Materials and methods

Construction of a Xenopus oocyte cDNA library

An adult *Xenopus* ovary was treated with collagenase (Boehringer Mannheim) to obtain oocytes free of follicle cells. Total RNA was extracted from the oocytes (~8.7 mg RNA from 5 ml of packed oocytes) by the acid guanidinium−thiocyanate−phenol−chloroform method (Chomczynski and Sacchi, 1987). From the poly(A)$^+$ RNA, a *Xenopus* oocyte cDNA library was constructed in λgt10.

Isolation of Xenopus M phase MAP kinase cDNA clones and DNA sequencing

Xenopus M phase-activated MAP kinase (~42 kDa) was purified from mature oocytes as described (Gotoh *et al.*, 1991). The kinase polypeptide was further purified by SDS−PAGE and digested with lysylendopeptidase and the peptides produced were separated on a Vydac C18 reverse-phase column. The separated peptides were sequenced by a gas-phase amino acid sequencer (470A Protein Sequencer, Applied Biosystems Japan, Tokyo) and five sequences were obtained: GYTK, MLTFNPHK, ALDLLDK, DVYIVQDLME and ARNYL. Two different mixed oligonucleotides corresponding to two of the peptides were synthesized: 5′-GA(T/C)GT(T/C/A//G)TA(T/C)AT(T/C/A)GT(T/C/A/G)CA(A/G)GA(T/C)(T/C)T(T/C/A/G)ATGGA-3′ and 5′-TACGA(T/C)TG(T/A/G)AA(A/G)TT(A/G)GG(T/A/G)-3′. Of the 6×10^4 recombinants, four clones were isolated as positive to both synthetic oligonucleotide probes. Two clones that contained 3.2 kb and 2.8 kb cDNA inserts, respectively, were subcloned into pUC18 and sequenced using standard dideoxynucleotide chain termination techniques with Sequenase™ (version 2, United States Biochemical) and [α-^{35}S]dATP. One of the clones (λXMK4) with a 3.2 kb insert covered the whole open reading frame. The other clone (λXMK8) contained part of the coding region and the overlapping sequence coincided with that of λXMK4.

Northern blotting

Total RNA was extracted from various tissues of *Xenopus* adult females as described above. RNA samples were glyoxylated and electrophoresed on an agarose gel in 10 mM sodium phosphate, pH 7.0, at 4 V/cm, transferred onto a Zeta-Probe membrane (Bio-Rad), and hybridized with an *Eco*RI fragment of *Xenopus* M phase MAP kinase cDNA (836th bp to 1497th bp) as a probe.

Preparation of anti-MAP kinase serum

The anti-MAP kinase serum was raised in rabbits against a peptide DMELDDLPKERLKE coupled to BSA through the N-terminal cysteine residue. This peptide sequence was derived from the near C-terminal sequence of ERK1 (Boulton *et al.*, 1990), and the corresponding sequence (335−348 amino acid residues) of *Xenopus* MAP kinase is very similar (12/14 identical). Booster injections of the peptide were given at 2-week intervals in complete adjuvant. The antiserum reacted with both purified mammalian MAP kinase and purified *Xenopus* MAP kinase (see text).

Preparation of oocyte extracts

Immature *Xenopus* oocytes (stage VI) were selected from 0.5 mg/ml collagenase-treated ovaries and cultured in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.41 mM CaCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4). Maturation was induced by treatment with 10 μM progesterone (Sigma). The germinal vesicle breakdown occurred 5−7 h after progesterone addition. Oocytes were homogenized in ~20 vols of a homogenization buffer (60 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1% aprotinin, 0.1 mM PMSF and 20 mM Tris−HCl, pH 7.5). The homogenate was centrifuged first at 5000 *g* for 5 min, and then at 300 000 *g* for 30 min. The supernatant was used as an extract for kinase assays. For Northern blotting, total RNA was extracted from the oocytes as described above.

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 3% gelatin in TBS (500 mM NaCl, 20 mM Tris, pH 7.5), membranes were incubated with the anti-MAP kinase antiserum at a dilution of 1:1000. Reacted proteins were detected by HRP−anti-rabbit IgG antibody.

Oocyte labeling and immunoprecipitation

Immature oocytes were prelabeled with 0.5 mCi/ml [^{32}P]orthophosphate in phosphate-free modified OR-2 (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 treated with progesterone, then extracts were prepared as described above. After incubation with 1/50 volume of the anti-MAP kinase antiserum at 0°C for 3 h in the presence of 0.2% SDS, immunocomplex was precipitated with protein A−Sepharose (Pharmacia) and washed with a solution containing 50 mM HEPES, 25 mM β-glycerophosphate, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 50 mM NaF, 1 mM DTT, 0.5% aprotinin, 0.1 mM PMSF and 1 mM Na_3VO_4. Immunoprecipitates were treated with Laemmli sample buffer, and analyzed by SDS−PAGE and autoradiography.

Kinase detection assays

Kinase activities of extracts toward exogenous MAP2 (final 20 μg/ml), MBP (Sigma, final 1 mg/ml) or histone H1 (Sigma, final 1 mg/ml) were assessed as described previously (Gotoh *et al.*, 1990b) except that 20 mM $MgCl_2$ and 1 μM protein kinase inhibitor peptide of cAMP-dependent protein kinase (Sigma) were present. MAP2 was purified from porcine brains (Nishida *et al.*, 1987). The kinase detection assay of MAP kinase in MBP-containing gel was performed as described previously (Kameshita and Fujisawa, 1989; Gotoh *et al.*, 1990a,b, 1991).

Purification of MPF and microinjection

Starfish MPF was purified from maturing oocytes by $p13^{suc1}$ affinity chromatography as described by Labbe *et al.* (1989) with a slight modification (E.Okumura and T.Kishimoto, in preparation). Microinjection of the purified MPF or the purified MAP kinase into immature oocytes was carried out in modified Barth's medium at 21°C with the microinjection apparatus (IM-1, Narishige, Tokyo).

Preparation of Xenopus cell-free extracts

Interphase extracts were prepared from eggs (metaphase II oocytes) sampled 40 min after activation by an electric shock (12 V, AC, 3 s) in MMR buffer (0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES, pH 7.8) by the method of Lohka and Maller (1985) and Felix *et al.* (1989) with a slight modification. Briefly, eggs were dejellied with 2% cysteine, pH 7.8, washed extensively with MMR at first and then twice with 0.1 M K-acetate, 2.5 mM Mg-acetate, 60 mM EGTA, 2 μg/ml cytochalasin B and 0.1 mM DTT. After excess buffer was removed, the eggs were crushed by centrifugation at 30 000 *g* for 15 min at 2°C. The cytoplasmic materials obtained were supplemented with ATP regeneration system (Felix *et al.*, 1989) and were further centrifuged at 300 000 *g* for 20 min at 2°C. The clear lysate was used as an interphase extract.

Acknowledgements

We are indebted to Drs T.Akiyama (Oosaka University), S.Hattori (National Institute of Neuroscience) and T.Kadowaki (University of Tokyo) for help and advice in producing the antiserum. We are grateful to F.Ito, H.Kosako and T.Yamashita for help.

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Received on April 15, 1991; revised on May 13, 1991