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Xenopus MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation

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***Xenopus* MAP kinase activator, a 45 kDa protein, has been shown to function as a direct upstream factor sufficient for full activation and both tyrosine and serine/threonine phosphorylation of inactive MAP kinase. We have now shown by using an anti-MAP kinase activator antiserum that MAP kinase activator is ubiquitous in tissues and is regulated post-translationally. Activation of MAP kinase activator is correlated precisely with its threonine phosphorylation during the oocyte maturation process. It is a key question whether MAP kinase activator is a kinase or not. We have shown that *Xenopus* MAP kinase activator purified from mature oocytes is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues. Dephosphorylation of purified activator by protein phosphatase 2A treatment inactivates its autophosphorylation activity as well as its activator activity. Thus, *Xenopus* MAP kinase activator is a protein kinase with specificity for both serine/threonine and tyrosine. Partial protein sequencing of purified activator indicates that it contains a sequence homologous to kinase subdomains VI and VII of two yeast protein kinases, STE7 and byr1.**

Key words: kinase cascade/MAP kinase/MAP kinase activator/serine/threonine/tyrosine kinase/signal transduction

Introduction

MAP kinases, originally described as 40–45 kDa serine/threonine kinases that are activated by various mitogenic stimuli (Ray and Sturgill, 1987, 1988; Hoshi *et al.*, 1988, 1989), are thought to be key molecules functioning in the growth factor- and differentiating factor-stimulated signaling pathways (Sturgill *et al.*, 1988; Ahn *et al.*, 1990; Boulton *et al.*, 1990, 1991; Gomez *et al.*, 1990; Gotoh *et al.*, 1990a,b; Miyasaka *et al.*, 1990; Alvarez *et al.*, 1991; Chung *et al.*, 1991; Pulverer *et al.*, 1991) and also in the M phase kinase cascade downstream of MPF (Pelech *et al.*, 1988; Sanghera *et al.*, 1990; Gotoh *et al.*, 1991a,b; Ferrell *et al.*, 1991; Posada *et al.*, 1991). MAP kinases are unique in requiring both tyrosine and serine/threonine phosphorylation to become active (Anderson *et al.*, 1990). Thus, it is a key question how the activation and phosphorylation of MAP kinases are induced in various signal transduction systems.

Recent studies have focused on direct upstream activators of MAP kinase. Activities that can induce phosphorylation and activation of MAP kinase have been detected in epidermal growth factor-stimulated 3T3 cells (Ahn *et al.*, 1991) and in nerve growth factor-stimulated PC12 cells (Gomez and Cohen, 1991). We have identified and purified a *Xenopus* MAP kinase activator from mature oocytes, whose molecular mass is 45 kDa (Matsuda *et al.*, 1992). It can be reasonably assumed that MAP kinase activators are protein kinases as they can induce phosphorylation of both phosphatase 2A and CD45-treated MAP kinases (Ahn *et al.*, 1991; Gomez and Cohen, 1991) as well as recombinant inactive MAP kinase (Matsuda *et al.*, 1992). However, previous studies failed to identify alternative substrates for phosphorylation by them besides MAP kinases (Ahn *et al.*, 1991; Matsuda *et al.*, 1992). Furthermore, inactive MAP kinases are capable of weak autophosphorylation on tyrosine and threonine/serine residues (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991; Matsuda *et al.*, 1992). These observations have raised the possibility that MAP kinase activator need not necessarily possess kinase activity, but may function by binding to MAP kinases, thereby enhancing their ability to undergo autophosphorylation. On the other hand, Posada and Cooper (1992) reported an activity in mature *Xenopus* oocytes that can phosphorylate kinase-defective mutants of MAP kinase. It is therefore of considerable importance to determine whether the direct upstream activators of MAP kinase are kinases or not. Here we have shown that a *Xenopus* 45 kDa activator is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues and thus is a protein kinase with specificity for both tyrosine and serine/threonine.

To reveal fully the MAP kinase activating pathway, it is necessary to know the activation mechanism of the activator. The activators have previously been shown to be deactivated by protein phosphatase 2A treatment (Gomez and Cohen, 1991; Matsuda *et al.*, 1992), suggesting the importance of phosphorylation of the activators for the activity. In this study, we produced a monospecific antibody to *Xenopus* 45 kDa activator and revealed that threonine phosphorylation of the activator is correlated with its activation, indicating that a direct upstream activating factor of MAP kinase activator may be a serine/threonine kinase.

Results

Production of anti-MAP kinase activator antiserum

We produced anti-*Xenopus* MAP kinase activator antiserum by immunizing rabbits with a synthetic peptide corresponding to the N-terminal 16 amino acid sequence of the *Xenopus* 45 kDa activator. The activator was purified to homogeneity from mature *Xenopus* oocytes as described previously

(Matsuda *et al.*, 1992) and was subjected to amino acid sequence analysis with gas phase sequencer to determine the N-terminal amino acid sequence. The antiserum obtained, but not preimmune serum, reacted strongly with the purified *Xenopus* activator (data not shown). The antibody specifically reacted with the 45 kDa activator in total extracts obtained from mature oocytes in the immunoblot analysis (Figure 1A, left), while the antibody preincubated with the N-terminal activator peptide did not react with any polypeptides (Figure 1A, right).

We then examined whether the MAP kinase activator activity was immunoprecipitable with this antiserum. The extracts prepared from mature oocytes were subjected to immunoprecipitation with the antiserum, the peptide-preabsorbed serum or preimmune serum and then each immunoprecipitate was assayed for MAP kinase activator activity by incubating each sample with recombinant inactive MAP kinase in the presence of ATP. It should be noted that in the absence of MAP kinase each immunoprecipitate did not possess MBP kinase activity (data not shown). Only the immunoprecipitate with the antiserum showed the MAP kinase activator activity, as revealed by activation of MBP kinase activity of recombinant MAP kinase in the kinase detection assay within MBP-containing gels (Figure 1B). These results indicated the specificity of the antiserum and confirmed the identity of MAP kinase activator as the 45 kDa protein.

The tissue distribution of MAP kinase activator was examined by immunoblotting. The 45 kDa MAP kinase activator was present in all the *Xenopus* tissues examined, with the highest concentration in brain (Figure 2).

Activation of MAP kinase activator is accompanied by its threonine phosphorylation during Xenopus oocyte maturation

Our previous study showed that MAP kinase activator is inactivated by phosphatase 2A treatment (Matsuda *et al.*, 1992). To examine the activation mechanism of MAP kinase activator, we followed the protein amount, activity and phosphorylation state of the 45 kDa activator during progesterone-induced oocyte maturation, using the anti-45 kDa activator antibody. There was no marked change in the protein amount, as revealed by the immunoblotting (Figure 3, top), while the immunoprecipitation experiment revealed a large increase in the MAP kinase activating activity during oocyte maturation (Figure 3, bottom). Thus, the activity of 45 kDa MAP kinase activator may be regulated post-translationally. The immunoprecipitation from ^{32}P-labeled oocytes demonstrated an increase in the phosphorylation level of the 45 kDa activator during this process (Figure 3, middle). The increase of the phosphorylation closely paralleled the increase of the activity of the 45 kDa activator (Figure 3, middle and bottom). The phosphorylation occurred mainly on threonine and partly on serine residues (Figure 4B). Almost complete dephosphorylation was induced by protein phosphatase 2A treatment (Figure 4A), which inactivated the activator activity of the 45 kDa activator (see Figure 6 and Matsuda *et al.*, 1992). These results indicate that MAP kinase activator may be activated by threonine phosphorylation.

MAP kinase activator is a serine/threonine/tyrosine kinase

To examine whether MAP kinase activator is a kinase or not, we first incubated purified *Xenopus* MAP kinase activator with [α-^{32}P]8-azido-ATP. The 45 kDa activator was radiolabeled only when the reaction mixture was exposed to UV light (Figure 5A) and the labeling was inhibited in the presence of excess ATP (Figure 5A). This indicates that the 45 kDa activator is an ATP-binding protein and may be a kinase.

To detect possible autophosphorylation, we incubated the purified activator with [γ-^{32}P]ATP. The 45 kDa activator itself became phosphorylated clearly (Figure 5B, lane 3). The phosphorylation went on linearly for at least 20 min at 30°C under the conditions used (data not shown). Surprisingly, the autophosphorylation occurred on serine, threonine and tyrosine residues (Figure 5C). The activator used was highly purified (Figure 5B, lane 2) and the autophosphorylation on serine, threonine and tyrosine residues was reproducibly detected in different preparations of the activator. Moreover, elution of the apparent autophos-

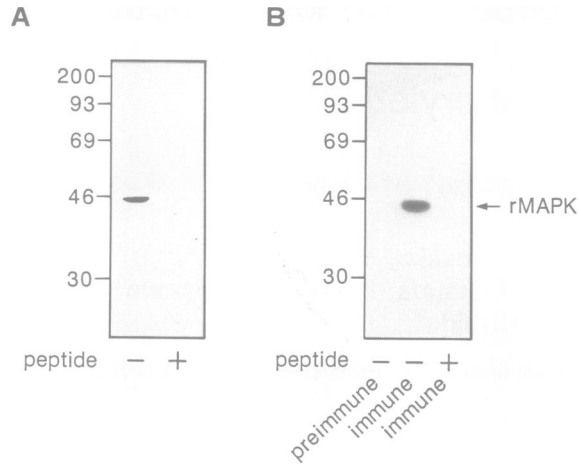

Fig. 1. Reactivity of anti-*Xenopus* MAP kinase activator antibody. (**A**) Extracts were prepared from mature *Xenopus* oocytes as described (Gotoh *et al.*, 1991a) and electrophoresed on a 10% acrylamide gel and immunoblotted with anti-*Xenopus* MAP kinase activator antibody that had been preincubated with (right lane) or without (left lane) 2 mg/ml antigen-peptide (the N-terminal activator peptide) for 2 h at 4°C. (**B**) Extracts obtained from mature oocytes (100 μl) were subjected to immunoprecipitation with preimmune serum (left lane) or immune serum that had been preincubated with (right lane) or without (middle lane) 5 mg/ml antigen-peptide for 2 h at 4°C. Then MAP kinase activator activity of the immunoprecipitates was measured as described in Materials and methods.

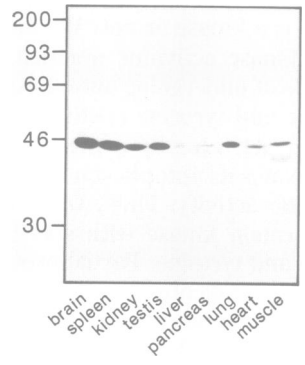

Fig. 2. Tissue distribution of MAP kinase activator. Extracts from various tissues (each 10 μg of protein) were electrophoresed and immunoblotted with anti-activator antibody.

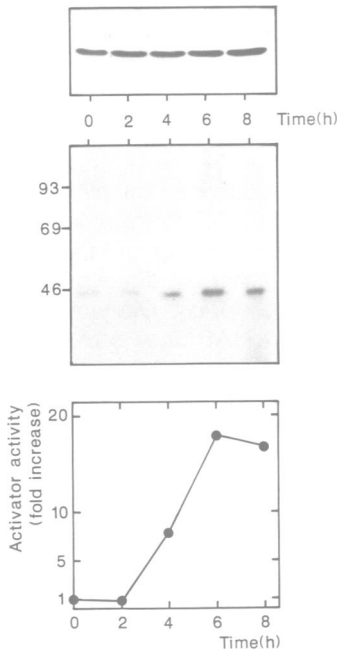

Fig. 3. Maturation-induced phosphorylation and activation of MAP kinase activator. Immature *Xenopus* oocytes (stage VI) were prepared as described (Gotoh *et al.*, 1991b) and divided in half. One half was prelabeled with 0.5 mCi/ml [^{32}P]orthophosphate in phosphate-free modified OR-2 for 3 h and the other half was not labeled. Maturation was induced by treatment with 10 μM progesterone (Sigma) and the extracts were prepared after indicated times. Nonlabeled extracts (10 μl) were electrophoresed and immunoblotted with anti-activator antibody (top). Labeled extracts were immunoprecipitated with anti-activator serum and autoradiographed after electrophoresis (middle). Nonlabeled extracts (40 μl) were immunoprecipitated with anti-activator serum and the immunoprecipitate was incubated with recombinant MAP kinase and ATP as described in Materials and methods. The MAP kinase activator activity was then quantified by measuring MBP kinase activity of the recombinant MAP kinase within MBP-containing gels with an image analyzer (FUJIX BAS2000) (bottom).

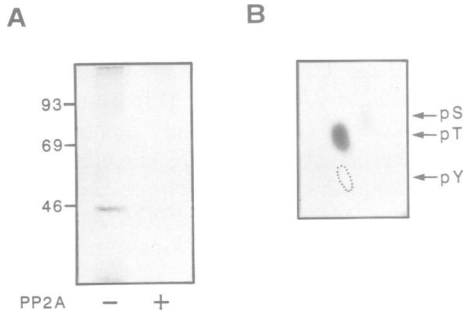

Fig. 4. MAP kinase activator is threonine phosphorylated *in vivo*. (**A**) Extracts were prepared from the progesterone-treated (6 h) oocytes labeled with [^{32}P]orthophosphate and subjected to immunoprecipitation as before. The immunoprecipitate was divided in half and one half was incubated for 20 min at 30°C with the catalytic subunit of phosphatase 2A (1 μg/ml) (right lane). The other half was incubated without phosphatase 2A (left lane). After electrophoresis, bands were detected by autoradiography. (**B**) The 45 kDa band in the left lane in A was excised and two-dimensional phosphoamino acid analysis was performed as described (Boyle *et al.*, 1991).

phorylation activity on the 45 kDa activator protein coincided with elution of the activator activity, namely that of the 45 kDa activator, on Mono-S chromatography in the final step for purification of MAP kinase activator (data not

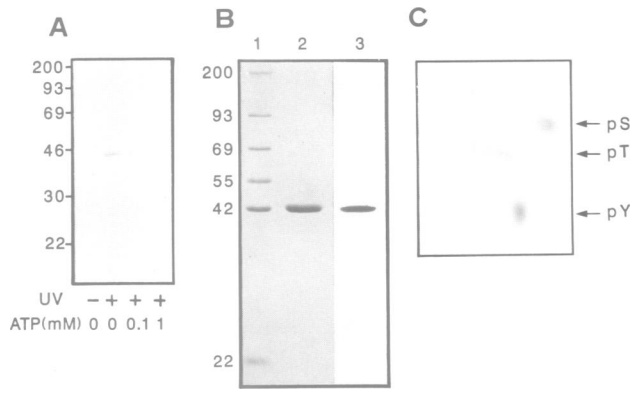

Fig. 5. Photoaffinity labeling and autophosphorylation of MAP kinase activator. (**A**) *Xenopus* MAP kinase activator was purified as described previously (Matsuda *et al.*, 1992). The purified activator was mixed with [α-^{32}P]8-azido-ATP in the presence of 0, 0.1 or 1 mM ATP and irradiated with UV lamp. Photolabeled proteins were analyzed by SDS−PAGE and autoradiography. Rainbow colored protein molecular weight markers (Amersham) were used. (**B**) The purified activator (2 μg) was incubated with [γ-^{32}P]ATP, electrophoresed, stained with Coomassie blue (lane 2) and autoradiographed (lane 3). Lane 1, molecular weight standards (200 kDa, myosin heavy chain; 93 kDa, phosphorylase b; 69 kDa, bovine serum albumin; 55 kDa, tubulin; 42 kDa, actin; 22 kDa, trypsin inhibitor). (**C**) The 45 kDa band on lane 2 in B was excised and subjected to phosphoamino acid analysis.

shown). Thus, the 45 kDa *Xenopus* MAP kinase activator is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues.

The autophosphorylation activity of the activator was inactivated by protein phosphatase 2A treatment (Figure 6, lower panel). The time-course of this inactivation process was coincident with that of inactivation of the activator activity by phosphatase treatment (Figure 6, upper panel). When the activity of phosphatase 2A was inhibited in the presence of okadaic acid during the treatment, there was no loss in autophosphorylation activity as well as activator activity of the 45 kDa MAP kinase activator (Figure 6). These results indicate that the autophosphorylation activity is correlated with the activator activity and suggest that the activator itself is responsible for the autophosphorylation.

To confirm further that the observed phosphorylation of the 45 kDa activator is due to an autophosphorylation reaction, we performed gel filtration chromatography. The purified activator was passed through a Sephadex G-100 column and each fraction was examined for the activator activity (the activity to phosphorylate and activate inactive MAP kinase) and the autophosphorylation activity. The activator activity (Figure 7A) and the apparent autophosphorylation (endogenous phosphorylation of the 45 kDa activator protein, Figure 7A, lower panel) co-eluted as a single, sharp symmetrical peak at the position corresponding to an apparent molecular mass of ~55 kDa (Figure 7A). This indicates that the activator exists as a monomer and rules out the possibility that another protein, for example a protein with M_r 45 kDa which is tightly associated with the activator is responsible for the activator phosphorylation or vice versa. Moreover, the extent of autophosphorylation was linearly correlated with the concentration of the activator (Figure 7B). In other words, the rate of autophosphorylation was independent of the activator concentration. This suggests that the phosphorylation may occur by an intramolecular reaction and thus further supports that the phosphorylation

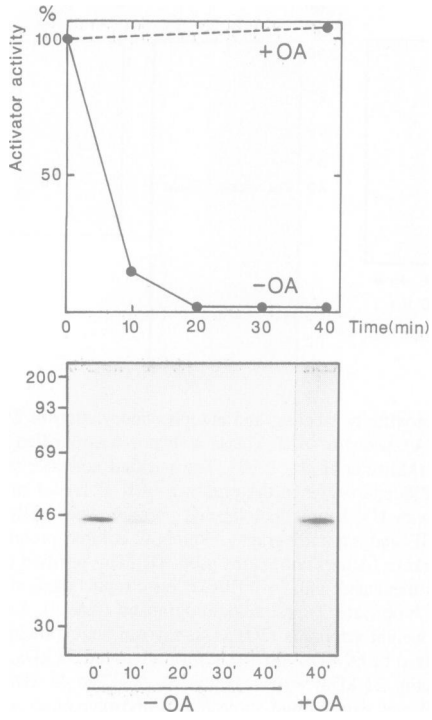

Fig. 6. Inactivation of the activator and autophosphorylation activity of MAP kinase activator by phosphatase 2A treatment. The purified activator was incubated for the indicated times at 30°C with phosphatase 2A (1 μg/ml) as described previously (Matsuda *et al.*, 1992). After the addition of okadaic acid (final 10 μM), the samples were assayed for the activator activity (upper) and for the autophosphorylation activity as described in Materials and methods (lower). In control incubations, okadaic acid was added simultaneously with phosphatase 2A and incubated for 40 min (broken line, +OA).

is an autophosphorylation. The peak fraction in the gel filtration chromatography was then subjected to immunoprecipitation with the anti-45 kDa activator antiserum, the N-terminal peptide-preabsorbed antiserum or preimmune serum. Only the immunoprecipitate with the antiserum exhibited the autophosphorylation activity (Figure 7C). Furthermore, the autophosphorylation again occurred on serine, threonine and tyrosine residues (Figure 7D). Taken together all of these results clearly indicate that *Xenopus* MAP kinase activator, a 45 kDa monomeric protein, is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues.

Partial protein sequencing of the purified 45 kDa activator after digestion with lysylendopeptidase (Figure 8) revealed that *Xenopus* activator contains an amino acid sequence corresponding to the consensus sequence of subdomains VI and VII of known serine/threonine kinases (Hanks *et al.*, 1988). This is consistent with our conclusion that *Xenopus* MAP kinase activator is a protein kinase. Interestingly, this sequence of *Xenopus* activator is highly homologous to subdomains VI and VII of two yeast protein kinases, byrl and STE7 (Figure 8). Xenopus activator is at least 76% identical (93% homologous) to byrl and is at least 66% identical (86% homologous) to STE7 in the subdomains VI and VII.

Discussion

MAP kinase is unique in that it becomes activated only when both tyrosine and serine/threonine residues are phosphorylated, and is thought to play key roles in the growth factor- and differentiating factor-stimulated signaling pathways and also in the M phase kinase cascade downstream of MPF (see Introduction). To understand these signal transduction pathways, it is of considerable importance to reveal the activation mechanism of MAP kinase. One of the key questions is whether the direct upstream activator of MAP kinase is a kinase or not. Our previous study identified and purified *Xenopus* MAP kinase activator with an apparent molecular mass of 45 kDa (Matsuda *et al.*, 1992). The 45 kDa activator has been shown to be sufficient for full activation and both tyrosine and serine/threonine phosphorylation of inactive MAP kinase (Matsuda *et al.*, 1992). In this study, we produced a specific anti-MAP kinase activator antibody, based on the N-terminal sequence of the 45 kDa activator. All the data obtained with this antibody and others presented here confirmed the identity of MAP kinase activator as the 45 kDa monomeric protein and have convincingly shown that the 45 kDa activator is a protein kinase with dual specificity for both tyrosine and serine/threonine. The immunoprecipitate obtained with the anti-45 kDa activator antiserum from the almost homogeneous monomeric 45 kDa activator fraction underwent autophosphorylation on serine, threonine and tyrosine residues (Figure 7). Previous studies have failed to identify alternative substrates for phosphorylation by the activator besides MAP kinase. By having shown the autophosphorylation activity of the activator, our study has revealed identity of MAP kinase activator as a serine/threonine/tyrosine kinase. As the purified 45 kDa activator alone can fully activate recombinant MAP kinase by inducing its phosphorylation on both tyrosine and threonine/serine residues (Matsuda *et al.*, 1992), we can suggest that *Xenopus* MAP kinase activator may function as a serine/threonine/tyrosine kinase in activating MAP kinase. Consistent with this suggestion, the serine/threonine/tyrosine autophosphorylation activity of the 45 kDa activator was inactivated in parallel with inactivation of its activator activity during phosphatase 2A treatment (Figure 6). In addition, Posada and Cooper (1992) recently demonstrated that a 0.1 M NaCl fraction obtained by DEAE-cellulose chromatography from mature oocytes, which presumably contained 45 kDa activator (Matsuda *et al.*, 1992), catalyzed tyrosine and threonine phosphorylations of kinase-inactive mutant of MAP kinase.

Although previous studies showed that phosphorylation of MAP kinase activator is required for its activity (Gomez and Cohen, 1991; Matsuda *et al.*, 1992), it was not known whether activator is activated by phosphorylation in cells. Here we have shown that activation of *Xenopus* MAP kinase activator is precisely correlated with its threonine phosphorylation during oocyte maturation and that phosphatase 2A treatment induces almost complete dephosphorylation as well as inactivation of the activator (Figures 3, 4 and 6). Therefore *Xenopus* MAP kinase activator may be activated by threonine phosphorylation, suggesting that a direct upstream activating factor for MAP kinase activator may be a protein serine/threonine kinase. As MPF could not

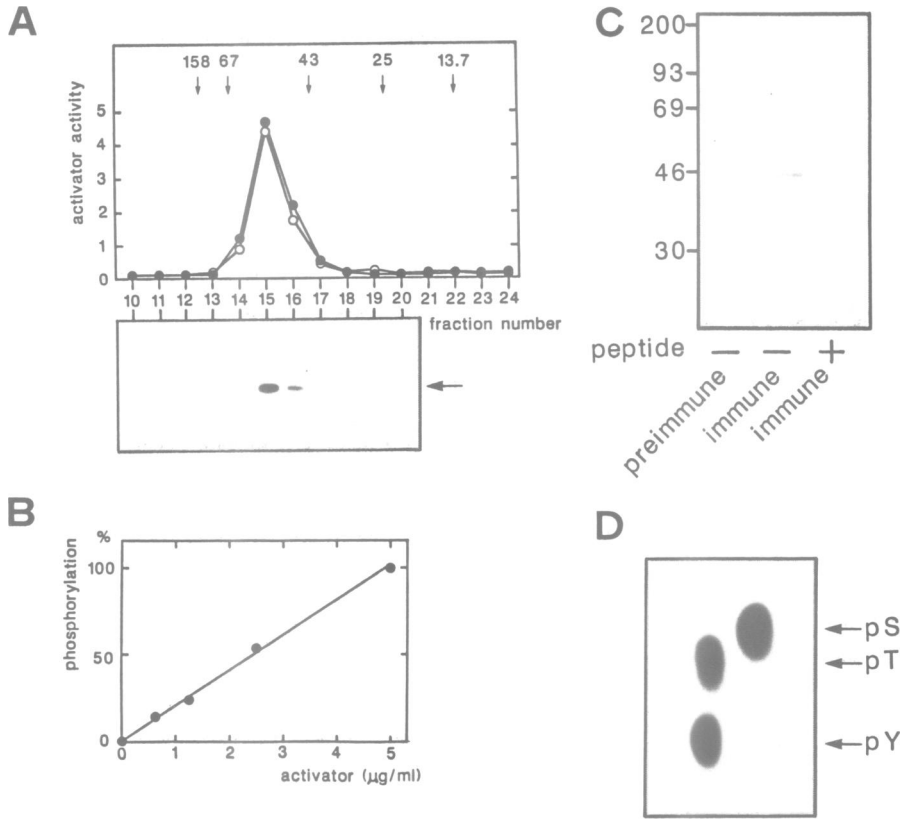

Fig. 7. Co-elution of the activator activity and autophosphorylation of MAP kinase activator upon gel filtration chromatography. (**A**) The purified activator (20 μg) was passed through a Sephadex G-100 column (1.2 × 35 cm) equilibrated with buffer A (50 mM Tris−Cl, pH 7.3, 1.5 mM EGTA, 0.2 M NaCl, 0.03% Brij 35, 2 mM DTT and 1 mM vanadate). Fractions were assayed for the activator activity as described (Matsuda *et al.*, 1992). The activities to phosphorylate recombinant MAP kinase (○) and to enhance the phosphorylation of MBP by recombinant MAP kinase (●) are shown in arbitrary units (top). Fraction numbers 10−20 were incubated with [γ-^{32}P]ATP as described in Materials and methods and analyzed by SDS−PAGE and autoradiography (bottom). An arrow denotes the electrophoretic position of the activator. (**B**) The peak fraction (number 15) from Sephadex G-100 in A was diluted with buffer A to 1, 2, 4 and 8-fold and incubated with [γ-^{32}P]ATP. After SDS−PAGE, the radioactivity of the 45 kDa band was quantified with an image analyzer (FUJIX BAS 2000). (**C**) The peak fraction (number 15) was immunoprecipitated with preimmune serum (left lane) or immune serum which had been preincubated with (right lane) or without (middle lane) 5 mg/ml antigen-peptide for 2 h at 4°C. The autophosphorylation of the immunoprecipitates was measured as described in Materials and methods. (**D**) The 45 kDa band on middle lane in C was excised and subjected to phosphoamino acid analysis.

reactivate the activator previously inactivated by the phosphatase treatment (Matsuda *et al.*, 1992), MPF may not be the activator kinase. Identification of this putative protein kinase will be the next crucial step for our understanding of the MAP kinase activation pathways.

Our preliminary sequencing data revealed striking similarities between *Xenopus* MAP kinase activator and two yeast protein kinases, byrl and STE7, in kinase subdomains VI and VII (Figure 8). Sequences of other peptide fragments obtained from *Xenopus* activator can also find corresponding sequences of byrl and STE7 (unpublished data). byrl, a *Schizosaccharomyces pombe* protein kinase, is supposed to function downstream of rasl and is involved in the sexual differentiation pathway (Nadin-Davis and Nasim, 1988, 1990). A putative *S.pombe* homolog of MAP kinase, spkl, has also been shown to be critical for the mating pathway (Toda *et al.*, 1991). Thus, byrl may function as an upstream activator of spkl. STE7 is a *Saccharomyces cerevisiae* protein kinase functioning in the mating pheromone-induced signaling pathway (Teague *et al.*, 1986). Putative *S.cerevisiae* homologs of MAP kinase, FUS3 and KSS1, function also in the mating signal transduction (Courchesne

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STE7        IIHRDIKPSNVLINSKGQIKLCDFGVSKK
            |•|||•||||•|•||•|• || |||||
activator   IMHRDVKPSNILVNSRGEXKLXDFGVSGQ
            |•|||•||||••|||||| || |||||||•
byrl        IIHRDLKPSNVVVNSRGEIKLCDFGVSGE
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Fig. 8. Partial amino acid sequence of *Xenopus* MAP kinase activator perfectly matches the consensus sequence of serine/threonine kinase subdomains VI and VII. The purified activator was digested with lysylendopeptidase, and the three peptide fragments separated by HPLC column were subjected to amino acid sequence analysis. These sequences were compared to those of STE7 and byrl (subdomains VI and VII). X indicates an unidentified amino acid. Emphasized letters indicate conserved amino acids among serine/threonine kinases (Hanks *et al.*, 1988). Aligned amino acid residues identical between activator and STE or between activator and byrl are denoted by |, whereas ● represents homologous amino acid substitutions.

et al., 1989; Elion *et al.*, 1990). Thus, STE7 may be an upstream direct activator of FUS3 and/or KSS1. Our recent study has demonstrated that mammalian MAP kinase activator in epidermal growth factor-stimulated cells is homologous to *Xenopus* activator characterized here in terms of physicochemical, immunological and functional properties

(K.Shirakabe, Y.Gotoh and E.Nishida, in preparation). Therefore the MAP kinase activator/MAP kinase system may play a key role in a variety of intracellular signal transduction pathways in yeasts to vertebrates.

Materials and methods

Production of anti-MAP kinase activator antibody

The anti-activator serum was raised in rabbits against a synthetic peptide corresponding to the N-terminal 16 amino acid sequence of *Xenopus* MAP kinase activator, PKKKPTPIQLNPNPEG. The peptide was coupled to bovine serum albumin through the N-terminal cysteine residue. The N-terminal sequence of the purified 45 kDa activator (Matsuda *et al.*, 1992) was determined by a gas phase sequencer. Two booster injections were given at 2-week intervals. The antibody was affinity-purified on the peptide coupled to the AF-aminotoyopearl (TOSOH) according to the manufacturer's instructions. Immunoblot analysis with the antibody (1:100 dilution) was performed as previously described (Matsuda *et al.*, 1992; Gotoh *et al.*, 1991b).

Immunoprecipitation

Samples were incubated with 1/20 volume of the anti-activator serum at 4°C for 2 h and then immunocomplex was precipitated with protein A−Sepharose (Pharmacia) and washed three times with TBST (20 mM Tris−Cl, pH 7.5, 500 mM NaCl and 0.05% Tween 20). To detect the MAP kinase activator activity, immunoprecipitates were incubated for 20 min at 30°C with 10 μg/ml recombinant *Xenopus* MAP kinase and 50 μM ATP in 20 mM Tris−Cl, pH 7.5, 15 mM $MgCl_2$ and 2 mM EGTA in a final volume of 30 μl. The reaction was stopped by the addition of Laemmli's sample buffer and subsequently subjected to the kinase detection assay within MBP-containing gels (Gotoh *et al.*, 1990b). To detect autophosphorylation of the activator (Figures 7C and D), immunoprecipitates were suspended in 20 mM Tris−Cl, pH 7.5, 150 mM NaCl and 3 mM $MnCl_2$ and incubated for 60 min at 30°C with 2 μM [γ-^{32}P]ATP (10 μCi) in a final volume of 30 μl. The reaction was stopped by washing with ice-cold TBST and then Laemmli's sample buffer was added. After electrophoresis, bands were detected by autoradiography.

Preparation of extracts from various tissues

Various tissues of *Xenopus* adult females were homogenized in 60 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM NaF, 1 mM Na_3VO_4, 20 μg/ml aprotinin, 1 mM PMSF, 20 mM Tris−Cl, pH 7.5 and 1% Triton X-100. The homogenate was centrifuged at 10 000 *g* for 20 min and Laemmli's sample buffer was added to the supernatant.

Photoaffinity labeling

The purified activator (0.3 μg) was mixed with 12 μM [α-^{32}P]8-azido-ATP (10.9 Ci/mmol) in the presence of 10 mM $MgCl_2$ and 0, 0.1 or 1 mM ATP in a final volume of 30 μl. The sample was irradiated with UV lamp (254 nm) at a distance of 2 cm for 5 min at 0°C and the reaction was stopped by the addition of Laemmli's sample buffer and boiling.

Autophosphorylation of MAP kinase activator

Samples for assay were incubated for 15 min at 30°C with 20 μM [γ-^{32}P]ATP (5 μCi) in 20 mM Tris−Cl (pH 7.5), 10 mM $MgCl_2$ and 2 mM EGTA in a final volume of 30 μl. The reaction was stopped by the addition of Laemmli's sample buffer and boiling.

Partial amino acid sequence

The purified activator (60 μg) was subjected to SDS−PAGE and transferred to PVDF membrane. Following staining with Ponceau S, the 45 kDa band was excised and digested with *Achromobacter* protease I. The peptide fragments were separated by HPLC using a μ-Bondasphere 5C8-300 (2.1 × 150 mm) column and subjected to amino acid sequence analysis with gas phase sequencer (Applied Biosystems model 470A).

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