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cDNA cloning of MAP kinase kinase reveals kinase cascade pathways in yeasts to vertebrates

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A *Xenopus* 45 kDa protein has been identified as an immediate upstream factor sufficient for full activation of MAP kinase, and is shown to be capable of undergoing autophosphorylation on serine, threonine and tyrosine residues. In this study, we show that purified 45 kDa protein can phosphorylate a kinase-negative mutant of *Xenopus* MAP kinase on tyrosine and threonine residues, suggesting that the 45 kDa protein functions as a MAP kinase kinase to activate MAP kinase. We then report the cloning and sequencing of a full-length cDNA encoding this 45 kDa MAP kinase kinase, and show that it is highly homologous to four protein kinases in fission and budding yeasts: byr1, wis1, PBS2 and STE7. These yeast kinases are therefore suggested to function as a direct upstream activator for a presumed MAP kinase homolog in each signal transduction pathway involved in the regulation of cell cycle progression or cellular responses to extracellular signals. Finally, we report bacterial expression of recombinant MAP kinase kinase that can be phosphorylated and activated by *Xenopus* egg extracts.

Key words: kinase cascade/MAP kinase activator/MAP kinase kinase/signal transduction/yeast homologs

Introduction

MAP kinases (reviewed in Cobb *et al.*, 1991; Sturgill and Wu, 1991; Nishida and Gotoh, 1992; Pelech and Sanghera, 1992) have been implicated in a variety of signal transduction pathways, including the growth factor-stimulated signaling process and the M phase promoting factor (MPF)-induced kinase cascade (Ray and Sturgill, 1987; Hoshi *et al.*, 1988; Pelech *et al.*, 1988; Sturgill *et al.*, 1988; Gotoh *et al.*, 1990a,b, 1991a,b; Alvarez *et al.*, 1991; Boulton *et al.*, 1991; Posada *et al.*, 1991; Pulverer *et al.*, 1991). MAP kinases are unique in requiring both tyrosine and threonine phosphorylations to become active (Anderson *et al.*, 1990). Thus, it is of considerable importance to reveal the activation mechanism of MAP kinase.

Previous studies detected activities that can induce activation and phosphorylation of MAP kinase in mammalian cultured cells (Ahn *et al.*, 1991; Gomez and Cohen, 1991). Several lines of evidence suggest the existence of an activity catalyzing the phosphorylation of MAP kinase on tyrosine and threonine residues (Gomez and Cohen, 1991; Nakielny *et al.*, 1992; Posada and Cooper, 1992). We have identified and purified a single, 45 kDa MAP kinase activator from *Xenopus* mature oocytes that induces phosphorylation and activation of recombinant MAP kinase (Matsuda *et al.*, 1992). This 45 kDa activator is shown to function as a key intermediate in the MPF-induced kinase cascade (Matsuda *et al.*, 1992). Most recently, we have shown that the 45 kDa activator is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues (Kosako *et al.*, 1992). Thus, it is a key question whether or not the 45 kDa MAP kinase activator functions as a kinase to activate MAP kinase. In this report, we showed first that the purified 45 kDa activator can phosphorylate a kinase-negative mutant of MAP kinase and a wild-type MAP kinase to a similar extent on both tyrosine and threonine residues, thus indicating that the MAP kinase activator should be called MAP kinase kinase (MAPKK). Then, we carried out molecular cloning of this 45 kDa MAPKK. Sequencing of this cDNA has revealed a very intriguing fact: *Xenopus* MAPKK is highly homologous to several yeast kinases implicated in a variety of signal transduction pathways. Finally, we expressed this *Xenopus* MAPKK cDNA in *Escherichia coli* and purified it. This recombinant MAPKK underwent autophosphorylation, although weak, on serine, threonine and tyrosine residues, and was phosphorylated and activated by a fraction obtained from *Xenopus* egg extracts. Thus, this study, by having cloned a MAPKK cDNA for the first time, has revealed the existence of a common kinase cascade of MAPKK/MAP kinase in yeasts to vertebrates.

Results

A 45 kDa MAP kinase activator can function as MAP kinase kinase

To examine whether purified 45 kDa *Xenopus* MAP kinase activator (Figure 1A, lane 4) can phosphorylate MAP kinase without the aid of the autophosphorylation activity of MAP kinase (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991; Matsuda *et al.*, 1992), we produced a kinase-negative mutant of *Xenopus* MAP kinase by changing a lysine at the ATP-binding site to aspartic acid. Both the mutant and the wild-type MAP kinases were expressed as glutathione-S-transferase (GST)-fusion proteins and purified on a glutathione (GSH)-agarose affinity column (Figure 1A, lanes 2 and 3). The wild-type MAP kinase was capable of undergoing autophosphorylation (Figure 1B, upper, lane 1), whereas the mutant was completely inactive (Figure 1B, upper, lane 2). Phosphorylation of the wild-type MAP kinase induced by the 45 kDa activator was ~10-fold greater than autophosphorylation of the MAP kinase (Figure 1B, upper), and after preincubation with the activator the MBP-phosphorylating activity of the wild-type MAP kinase was increased >100-fold (Figure 1B, lower). The mutant MAP kinase was completely inactive in the kinase activity even after incubation with the 45 kDa activator and ATP (Figure 1B, lower, lane 5). However, the mutant MAP kinase was

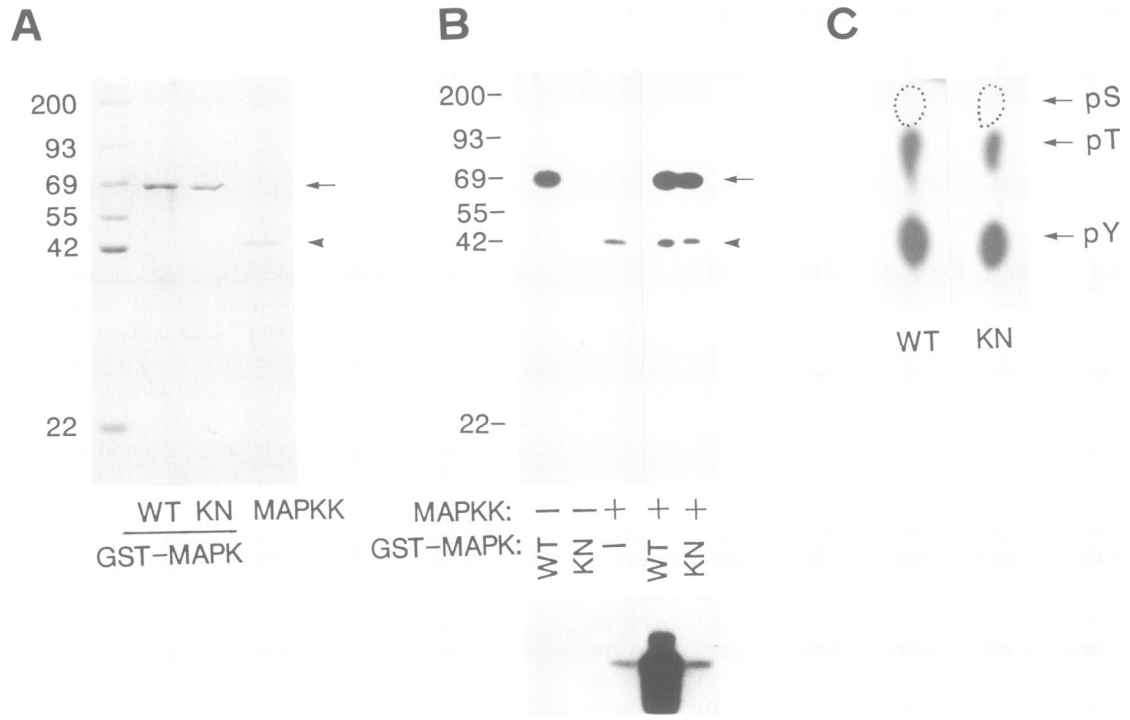

Fig. 1. Phosphorylation of a kinase-negative mutant of MAP kinase (MAPK) by purified *Xenopus* 45 kDa activator (MAPKK). (**A**) A kinase-negative (KN) mutant of *Xenopus* MAPK was produced by changing a lysine at the ATP-binding site to aspartic acid. Both the wild-type (WT) and the mutant (KN) MAPKs were expressed in *E.coli* as a GST-fusion protein, purified on a GSH−agarose affinity column and electrophoresed. Purified *Xenopus* 45 kDa MAPKK was also electrophoresed. The gel was stained with Coomassie blue. An arrow denotes the position of GST−MAPK and an arrowhead the MAPKK. (**B**) Purified MAPKs (WT and KN, 50 μg/ml) were incubated with 50 μM [γ-^{32}P]ATP (the first two lanes 10 μCi; other lanes, 1 μCi) in the absence (−) or presence (+) of purified 45 kDa MAPKK (5 μg/ml). After electrophoresis, the gel was autoradiographed (upper). Phosphorylation of MBP by GST−MAPKs after the incubation with or without MAPKK was examined as described in Materials and methods (lower). (**C**) The WT- and KN-MAPKs that were phosphorylated by the activator in (B) were excised and subjected to phosphoamino acid analysis.

phosphorylated by purified 45 kDa activator in a manner similar to the wild-type MAP kinase (Figure 1B, upper, lanes 4 and 5). Both tyrosine and threonine residues were phosphorylated (Figure 1C). These results suggest that purified 45 kDa MAP kinase activator functions as a protein tyrosine/threonine kinase to activate MAP kinase. We call the MAP kinase activator MAP kinase kinase (MAPKK) hereafter.

cDNA cloning of Xenopus MAPKK

We carried out molecular cloning of *Xenopus* 45 kDa MAPKK. Purified MAPKK was digested with endoproteinase Lys-C or Asp-N and the amino acid sequences of 12 peptides were determined. An N-terminal 19 amino acid sequence was also determined. A series of degenerate oligonucleotides corresponding to two of the above fragments were used for polymerase chain reaction (PCR). An amplified product of ~1 kb was obtained. This PCR product was sequenced and found to contain 10 peptide sequences that were determined and then used to screen a *Xenopus* ovary cDNA library. A positive clone with a 2.1 kb cDNA insert contained a complete open reading frame which encodes a protein consisting of 395 amino acid residues with a calculated molecular mass of 43 740 Da (Figure 2). The predicted amino acid sequence contained all the conserved residues of protein kinases and 11 kinase subdomains (Hanks *et al.*, 1988). Furthermore, it contained all the amino acid sequences (106 residues) of 12 peptide fragments determined by microprotein sequencing (Figure 2). The deduced N-terminal sequence also matched completely the N-terminal sequence determined from purified protein (Figure 2). These data indicate unequivocally that the cDNA described here encodes *Xenopus* 45 kDa MAPKK. Northern blot analysis detected a major (~3.5 kb) MAPKK transcript in ovary and other tissues (Figure 3). The homology search revealed that the fission yeast *Schizosaccharomyces pombe* genes byr1 (Nadin-Davis and Nasim, 1988) and wis1 (Warbrick and Fantes, 1991), and the budding yeast *Saccharomyces cerevisiae* genes PBS2 (Boguslawski and Polazzi, 1987) and STE7 (Teague *et al.*, 1986) show striking similarities to *Xenopus* MAPKK (Figure 4). These four yeast kinases all encode a family of protein kinases and show a high degree of similarity to one another. *Xenopus* MAPKK is 45, 43, 41 and 39% identical to byr1, wis1, PBS2 and STE7, respectively, over their shared lengths. Within the kinase domain, the identity increased to 54, 52, 54 and 45%, respectively.

Bacterial expression of Xenopus MAPKK

We expressed recombinant *Xenopus* MAPKK as a GST-fusion protein and purified it (Figure 5A, lane 1). The expressed GST−MAPKK was recognized by anti-N-terminal peptide antibody (data not shown) which also reacted with mammalian MAPKK (Kosako *et al.*, 1992; Shirakabe *et al.*, 1992). Purified GST−MAPKK was capable of undergoing autophosphorylation (Figure 5A, lanes 2, 3 and 4), albeit weak, on serine, threonine and tyrosine residues (Figure 5B). The autophosphorylation reaction was

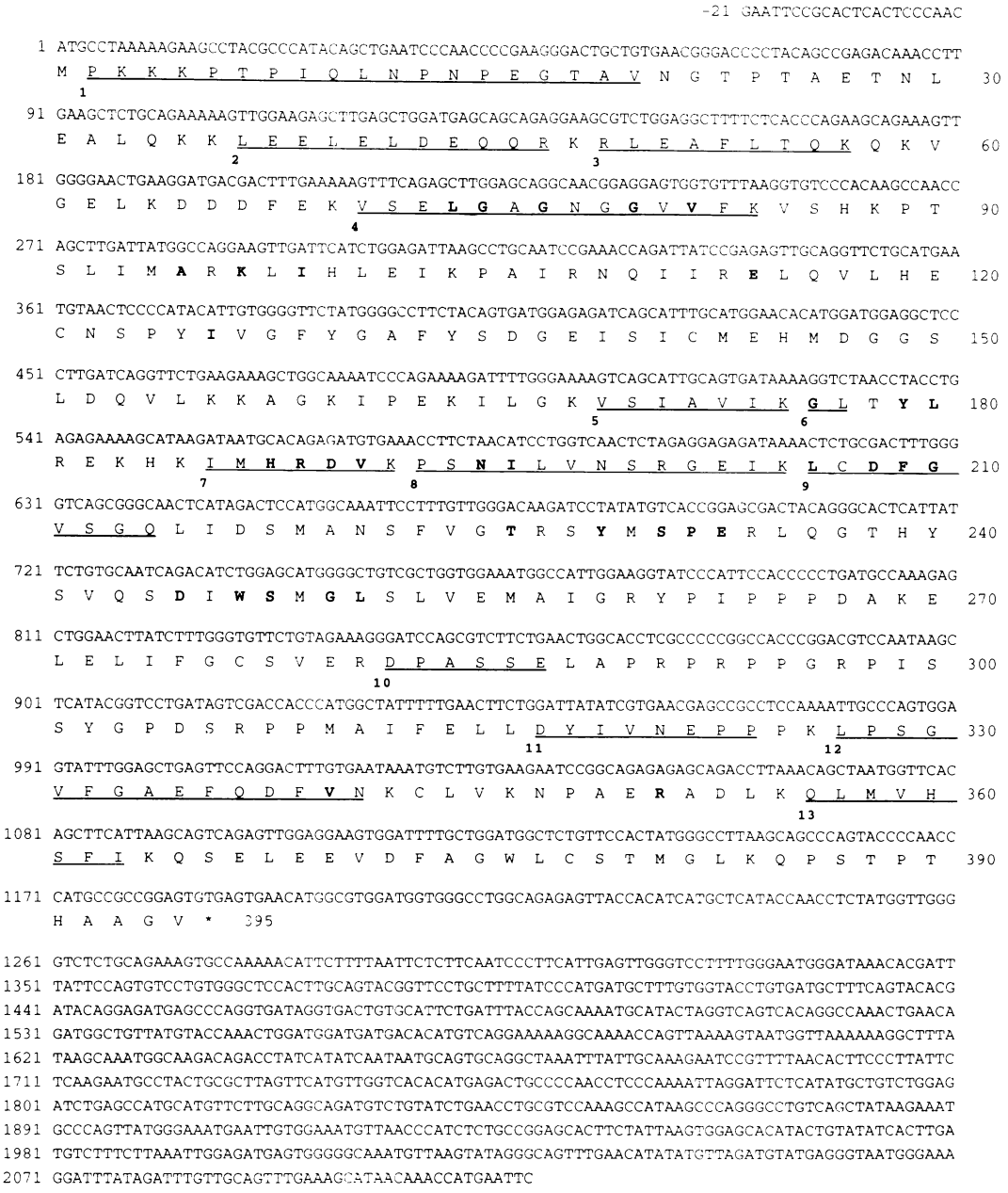

Fig. 2. cDNA cloning of *Xenopus* MAPKK. The nucleotide sequence of the *Xenopus* MAPKK cDNA and its predicted amino acid sequence are shown. Nucleotides and amino acids are numbered on the left and right, respectively. Peptide sequences derived from purified MAPKK are underlined and numbered (1−13). Questionable amino acid residues (I^{204}, C^{207}, S^{285}) in microprotein sequencing were verified by cDNA encoded protein sequence. The conserved amino acid residues among known protein kinases (Hanks *et al.*, 1988) are indicated by bold letters.

greater with Mn^{2+} than with Mg^{2+} (Figure 5A, lanes 2−4). Furthermore, the GST−MAPKK and 11aa−MAPKK, which was produced by cleaving the GST−MAPKK with Factor Xa, were both phosphorylated by a Q-Sepharose-adsorbed fraction of *Xenopus* egg extracts (Figure 6A). In addition, after this phosphorylation reaction both MAPKKs became active toward the kinase-negative MAP kinase (Figure 6B) and the wild-type MAP kinase (Figure 6C). The wild-type MAP kinase thus phosphorylated by the activated GST−MAPKK or 11aa−MAPKK became active toward MBP (Figure 6C). Therefore, the recombinant MAPKK can be phosphorylated and activated by a fraction obtained from *Xenopus* M phase extracts. These data confirmed that the *Xenopus* MAPKK whose cDNA has been cloned here is a protein kinase with dual specificity for both tyrosine and serine/threonine residues.

Discussion

Our previous studies demonstrated unambiguously that a single 45 kDa protein is sufficient for full activation and phosphorylation of MAP kinase, and thus is identified as MAP kinase activator (Kosako *et al.*, 1992; Matsuda *et al.*, 1992). Recent studies from several laboratories suggested the existence of an enzyme(s) which can catalyze phosphorylation of MAP kinase on threonine and tyrosine residues (Gomez and Cohen, 1991; Nakielny *et al.*, 1992; Posada and Cooper, 1992). Therefore, it was an important question whether or not our 45 kDa activator acts as MAPKK. In this study, by using a kinase-negative mutant of MAP kinase, we have shown that purified 45 kDa activator can phosphorylate MAP kinase on both tyrosine and threonine residues without the aid of the auto-

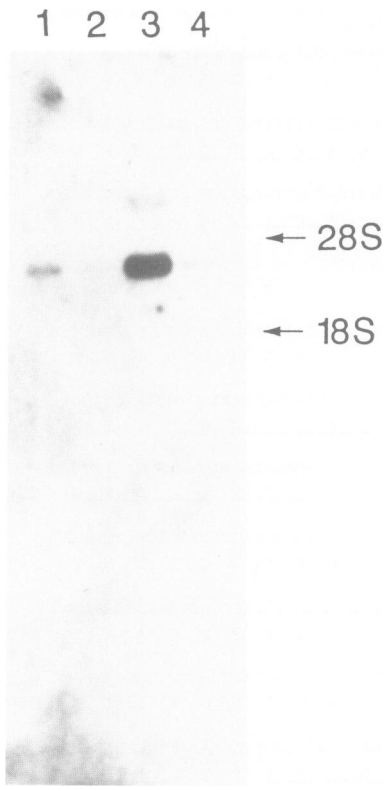

Fig. 3. Northern blot analysis of MAPKK. Ten micrograms each of total RNA extracted from *Xenopus* tissues were blotted with MAPKK cDNA probe. **Lane 1,** intestine; **lane 2**, spleen; **lane 3**, ovary; **lane 4**, lung. A major 3.5 kb MAPKK transcript was detected in the tissues examined.

phosphorylation activity of MAP kinase. This, together with our previous finding that the 45 kDa activator is capable of undergoing autophosphorylation on serine, threonine and tyrosine residues, indicates clearly that the 45 kDa activator, which should be called MAPKK, functions as a serine/threonine/tyrosine kinase.

Based on partial sequences of purified protein, we succeeded in cDNA cloning of MAPKK for the first time. This clone is indeed a MAPKK clone. It encodes a protein kinase with a calculated molecular mass (43.7 kDa) expected from purified MAPKK (45 kDa) and, importantly, bacterially expressed recombinant protein of this clone possesses the ability to phosphorylate and activate MAP kinase after incubation with *Xenopus* egg extracts.

Sequencing of MAPKK cDNA revealed that MAPKK is highly homologous to several yeast kinases. Since several putative yeast homologs of MAP kinase have already been reported, we can hypothesize the direct interaction between these putative yeast homologs of MAPKK and the putative yeast MAP kinases, as summarized in Figure 7. One of the putative *S.pombe* homologs of MAPKK, byr1 (Nadin-Davis and Nasim, 1988), is supposed to function downstream of ras1 (Fukui and Kaziro, 1985; Fukui *et al.*, 1986), an *S.pombe* homolog of vertebrate ras p21, and is essential to the sexual differentiation pathway. As a putative *S.pombe* homolog of MAP kinase, spk1 (Toda *et al.*, 1991) is also essential to the same signaling pathway, byr1 could be an immediate upstream of spk1 (Figure 7). It is noted that vertebrate ras p21 has been shown to function upstream of the MAPKK−MAP kinase system (Hattori *et al.*, 1992; Leevers and Marshall, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). In *S.cerevisiae*, STE7 (Teague *et al.*, 1986), a putative MAPKK homolog, is shown to function in the mating pheromone-response signaling pathway. STE7 may be a direct upstream of FUS3 (Elion *et al.*, 1990) and KSS1 (Courchesne *et al.*, 1989), putative MAP kinase homologs, as these genes also function in the mating signaling transduction. Another protein kinase, STE11 (Rhodes *et al.*, 1990), is shown to function within this signal transduction pathway, and a putative *S.pombe* homolog of STE11, byr2 (Wang *et al.*, 1991), has been suggested to function upstream of byr1. Moreover, BCK1 (related to STE11 and byr2; Lee and Levin, 1992), BCK2 (related to STE7 and byr1; K.Irie *et al.*, in preparation) and SLT2 (MPK1) (a MAP kinase homolog; Torres *et al.*, 1991) have been shown to act in the same pathway downstream of PKC1 (K.S.Lee *et al.*, in preparation). This cascade also seems to be analogous to the vertebrate system, in which MAPKK/MAP kinase can be activated downstream of protein kinase C. We have previously shown that *Xenopus* MAPKK is activated by threonine/serine phosphorylation probably catalyzed by an upstream kinase, MAPKK kinase (Kosako *et al.*, 1992). It is suggested that MAPKK kinase might correspond to byr2, STE11 and BCK1 (Figure 7). wis1 (Warbrick and Fantes, 1991) and PBS2 (Boguslawski and Polazzi, 1987) are other yeast homologs of MAPKK. We can hypothesize that upstream and downstream genes corresponding to MAPKK kinase and MAP kinase, respectively, may exist in these signaling pathways.

It should be noted that threonine and tyrosine residues (a TEY sequence) which are phosphorylated in the active vertebrate MAP kinase (Payne *et al.*, 1991) are conserved in all the putative yeast homologs of MAP kinase. It can therefore be speculated that putative yeast homologs of MAPKK may act as a tyrosine/threonine kinase. In summary, by having revealed the whole sequence of the direct upstream of MAP kinase, MAPKK, we can suggest that the MAPKK/MAP kinase system is conserved among various signal transduction pathways from yeast to vertebrate.

Materials and methods

Site-directed mutagenesis and bacterial expression of MAP kinases

Wild-type *Xenopus* MAP kinase (WT-MAPK) was subcloned into pUC18 as described previously (Gotoh *et al.*, 1991b). Oligonucleotide mutagenesis (Lys57-Asp) of WT-MAPK was performed according to the method of Kunkel *et al.* (1987) using the mutagenic primer 5′-CGAGTTGCTATCGATAAAATCAGCCC-3′ to yield KN-MAPK. This mutagenesis was confirmed by the presence of a newly introduced *Cla*I site. WT- and KN-MAPKs were amplified by PCR with the 5′ primer 5′-CCGGATCCCCATGGCAGCGGCAGGAGCTGCGTCT-3′ and the 3′ primer 5′-CGGGATCCGTCAGTACCCTGGCTGGAATCTAGCG-3′. These primers introduced *Bam*HI sites just before the start codon and after the stop codon. The amplified MAPK fragments were cloned into the *Bam*HI site of an expression vector pGEX-2T. Glutathione-S-transferase (GST), GST-WTMAPK and GST-KNMAPK were expressed in *E.coli* strain NM522 by incubating with 0.1 mM IPTG for 12 h. The cells were pelleted, washed and homogenized with 50 mM Tris−HCl (pH 7.5), 2 mM $MgCl_2$, 2 mM EGTA, 0.3% NP40, 2 mM dithiothreitol, 1 mM PMSF, 30 μg/ml DNase I. After clarification by centrifugation, the lysate was incubated with GSH−agarose beads for 1 h at 4°C. The beads were washed with excess STE [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA]. The fusion protein was eluted with 10 mM GSH, 50 mM Tris (pH 8). Free GSH was removed by dialysis against STE for 1 h at 4°C. Expression of WT/KN MAPKs was confirmed by immunoblotting with anti-MAP kinase (Gotoh *et al.*, 1991b).

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MAPKK      MPKKKP TPIQLNPNPE GTAVNGTPTA E..TNLEALQ KKLEELELDE  44
byr1     MFKRRRNP KGLVLNPNAS VKSSDNDHKE ELINNQKSFE SNVEAFMEQC  48
wis1   ASRRGLNIPP TLKQAVSETP .......... .FSTFSDILD AKSGTLNFKN 296
PBS2   TEGPHDTVGT TPRTGNSNNS SNSGSSGGGG LFANFSKYVD IKSGSLNFAG 336
STE7   LSPSSTNSTP STIQGLSNIA .TPVENEHSI SLPPLEESLS PAAADL.... 173

MAPKK  QQRKRLEAFL TQKQKVGELK DDDFEKVSEL GAGNGGVVFK VSHKPTSLIM  94
byr1   AHMNRRPAW. .....ISDLD NSSLEVVRHL GEGNGGAVSL VKHR..NIFM  90
wis1   KAVLNSEGVN FSSGSSFRIN MSEIIKLEEL GKGNYGVVYK ALHQPTGVTM 346
PBS2   KLSLSSKGID FSNGSSSRIT LDELEFLDEL GHGNYGNVSK VLHKPTNVIM 386
STE7   ......KDTL SGTSNGNYIQ LQDLVQLGKI GAGNSGTVVK ALHVPDSKIV 217

MAPKK  ARKLIHLEI. KPAIRNQIIR ELQVLHECNS P.YIVGFYGA FY...SDGEI 139
byr1   ARKTVYVGS. DSKLQKQILR ELGVLHHCRS P.YIVGFYGA FQ...YKNNI 135
wis1   ALKEIRLSL. EEATFNQIIM ELDILHKAVS P.YIVDFYGA FF...VEGSV 391
PBS2   ATKEVRLEL. DEAKFRQILM ELEVLHKCNS P.YIVDFYGA FF...IEGAV 431
STE7   AKKTIPVEQN NSTIINQLVR ELSIVKNVKP HENIITFYGA YYNQHINNEI 267

MAPKK  SICMEHMDGG SLDQVLK... .......KA. ..GKIPEKIL GKVSIAVIKG 176
byr1   SLCMEYMDCG SLDAILR... .......EG. ..GPIPLDIL GKIINSMVKG 172
wis1   FICMEYMDAG SMDKLYA... .......G.. ..GIKDEGVL ARTAYAVVQG 427
PBS2   YMCMEYMDGG SLDKIYD... .......ESS EIGGIDEPQL AFIANAVIHG 471
STE7   IILMEYSDCG SLDKILSVYK RFVQRGTVSS KKTWFNELTI SKIAYGVLNG 317

MAPKK  LTYLREKHKI MHRDVKPSNI LVNS.RGEIK LCDFGVSGQL IDSMANSFVG 225
byr1   LIYLYNVLHI IHRDLKPSNV VVNS.RGEIK LCDFGVSGEL VNSVAQTFVG 221
wis1   LKTLKEEHNI IHRDVKPTNV LVNSN.GQVK LCDFGVSGNL VASISKTNIG 476
PBS2   LKELKEQHNI IHRDVKPTNI LCSANQGTVK LCDFGVSGNL VASLAKTNIG 521
STE7   LDHLYRQYKI IHRDIKPSNV LINS.KGQIK LCDFGVSKKL INSIADTFVG 366

MAPKK  TRSYMSPERL QGTH...... .YSVQSDIWS MGLSLVEMAI GRYPIPPPDA 268
byr1   TSTYMSPERI RGGK...... .YTVKSDIWS LGISIIELAT Q......... 255
wis1   CQSYMAPERI RVGGPTNGVL TYTVQADVWS LGLTILEMAL GAYPYPPESY 526
PBS2   CQSYMAPERI KSLNPDRA.. TYTVQSDIWS LGLSILEMAL GRYPYPPETY 569
STE7   TSTYMSPERI QGN....... VYSIKGDVWS LGLMIIELVT GEFPLGGHND 409

MAPKK  KELELIFGCS VERDPASSEL APRPRPPGRP ISSYGPDSRP PMAIFELLDY 318
byr1   ...ELPWSFS NIDD...... .......... .......... SIGILDLLHC 276
wis1   T......... .......... .......... .......... ..SIFAQLSA 535
PBS2   D......... .......... .......... .......... ..NIFSQLSA 578
STE7   T......... .......... .......... .......... PDGILDLLQR 420

MAPKK  IVNEPPPKLP SG.VFGAEFQ DFVNKCLVKN PAERADLKQL MVHSFIKQS. 366
byr1   IVQEEPPRLP SS..FPEDLR LFVDACLHKD PTLRASPQQL CAMPYFQQA. 323
wis1   ICDGDPPSLP .D.SFSPEAR DFVNKCLNKN PSLRPDYHEL ANHPWLLKY. 582
PBS2   IVDGPPPRLP SD.KFSSDAQ DFVSLCLQKI PERRPTYAAL TEHPWLVKY. 626
STE7   IVNEPSPRLP KDRIYSKEMT DFVNRCCIKN ERERSSIHEL LHHDLIMKYV 470

MAPKK  .ELEEVDFAG WLCSTMGLKQ PSTPTHAAGV * 395
byr1   .LMINVDLAS WASNFRSS*  340
wis1   .QNADVDMAS WAKG...... .ALKEKGEKR S* 605
PBS2   .RNQDVHMSE YITERLERRN KILRERGENG LSKNVPALHM GGYSVNIQIK 675
STE7   SPSKDDKFRH WCRKIKSKIK EDKRIKREAL DRAKLEKKQS ERSTH* 515
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Fig. 4. Comparison of *Xenopus* MAPKK with four yeast kinases. The protein sequences of *Xenopus* MAPKK, *S.pombe* protein kinases byr1 and wis1, and *S.cerevisiae* protein kinases PBS2 and STE7 are aligned using the BESTFIT program from the UWGCG package. Dots indicate spaces introduced to maximally align sequences. Amino acids identical to MAPKK are shaded. Unlike the yeast kinases or other known kinases (Hanks *et al.*, 1988), MAPKK contains a relatively long insertion (~40 amino acid residues) between kinase subdomains IX and X.

Phosphorylation of MAP kinases

Xenopus MAP kinase activator (MAPKK, 45 kDa) was purified by sequential chromatography on DEAE–cellulose, heparin–Sepharose, hydroxylapatite and Mono-S as described previously (Matsuda *et al.*, 1992). GST–MAPKs were incubated in a solution containing 1 mM $MnCl_2$, 10 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP with or without MAPKK for 90 min at 25°C. Samples were electrophoresed and autoradiographed. GST alone was not phosphorylated by MAPKK at all. The bands of phosphorylated GST–MAPKs were excised from the gel and phosphoamino acid analysis was performed as described previously (Kosako *et al.*, 1992). To measure the myelin basic protein (MBP)-phosphorylating activity of GST-MAPKs, the incubation was carried out as above except that radiolabeled ATP was omitted. MBP (final

0.1 mg/ml) and [γ-^{32}P]ATP (0.5 μCi) were then added and the incubation was continued for another 10 min at 25°C. The reaction was stopped by the addition of Laemmli's sample buffer and boiling. After electrophoresis, the radioactivity of MBP bands was detected by autoradiography.

Peptide microsequencing

Purified *Xenopus* MAPKK was subjected to SDS−PAGE and transferred to PVDF membrane. The 45 kDa band was digested with endoproteinase Lys-C or endoproteinase Asp-N. The peptide fragments were separated by HPLC using a μ-Bondasphere 5C8-300 column and 12 peaks were subjected to amino acid sequence analysis with gas phase sequencer. The N-terminal sequence was also determined.

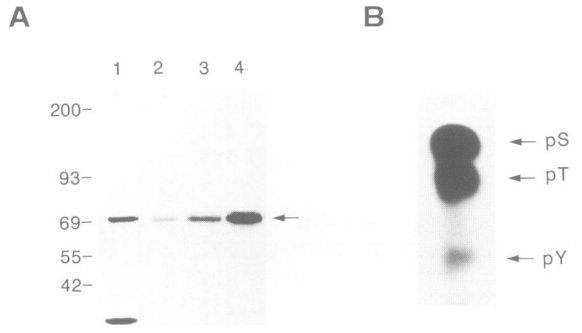

Fig. 5. Production of recombinant MAPKK in bacteria.
(**A**) Recombinant MAPKK was expressed in *E.coli* as a GST-fusion protein and purified by sequential chromatography on GSH−agarose and phenyl−Sepharose. Purified GST−MAPKK (protein staining, lane 1) was subjected to autophosphorylation reaction in the presence of 2 mM Mg^{2+} (lane 2), 20 mM Mg^{2+} (lane 3) or 2 mM Mn^{2+} (lane 4). An arrow denotes the position of GST−MAPKK. Lanes 2, 3 and 4 are autoradiographs. (**B**) The GST−MAPKK band in lane 4 in (A) was excised and subjected to phosphoamino acid analysis.

cDNA cloning and sequencing

The following oligonucleotides, derived from the peptides 1 and 11 (see Figure 2), were used as primers: 5′-AA(A/G)AA(A/G)CC(T/C/A/G)AC-(T/C/A/G)CC(T/C/A/G)AT(T/C/A)CA-3′ and 5′-GG(T/C)TC(A/G)-TT(T/C/A/G)AC(T/A/G)AT(A/G)TA(A/G)TC-3′. The first strand cDNA was synthesized from 4 μg of total RNA from *Xenopus* stage VI oocytes using SuperScript reverse transcriptase (BRL) with oligo(dT) primer. A mixture of an aliquot of cDNA and 500 pmol of each primer was preheated for 10 min at 94°C. PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) using the following parameters: 1 min at 94°C, 2 min at 47°C and 3 min at 72°C for 30 cycles. The resulting 0.95 kb PCR product was subcloned using TA cloning (Invitrogen), labeled with [α-^{32}P]dCTP by random priming (Boehringer Mannheim) and used to screen a *Xenopus* ovary cDNA library in λgt10 (Gotoh *et al.*, 1991b). Eight positive clones were isolated at a rate of ~$1/10^4$ phage. A 2.1 kb cDNA insert of clone No. 3 was subcloned into Bluescript (Stratagene) and sequenced by the standard dideoxynucleotide chain termination techniques with Sequenase (version 2.0, USB).

Northern blot analysis

Total RNA was extracted from adult *Xenopus* tissues by the acid guanidium thiocyanate−phenol−chloroform method (Chomczynski and Sacchi, 1987). For Northern analysis, RNA (~10 μg) was denatured in formamide/formaldehyde, electrophoresed through a 1.2% agarose gel in MOPS−formaldehyde and transferred to a nylon membrane. The membrane was prehybridized for 4 h in 40% formamide, 5 × SSCP, 10 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA. Random primer-generated [^{32}P]dCTP-labeled DNA probe (*Eco*RI fragment of clone No. 3, see above) was then added to the same solution for 15 h at 42°C. The membrane was then washed for 15 min each in 2 × SSC, 0.1% SDS, in 0.5 × SSC, 0.1% SDS, and in 0.2 × SSC, 0.1% SDS at 65°C, and exposed to autoradiography.

Production and purification of recombinant MAPKK

To express the MAPKK in bacteria, MAPKK cDNA (2.1 kb *Eco*RI fragment) was subcloned into the *Eco*RI site of expression vector pGEX-3X. This construction introduced seven amino acids (NSALTPN) between GST and MAPKK. GST−MAPKK was expressed in NM522 in the presence

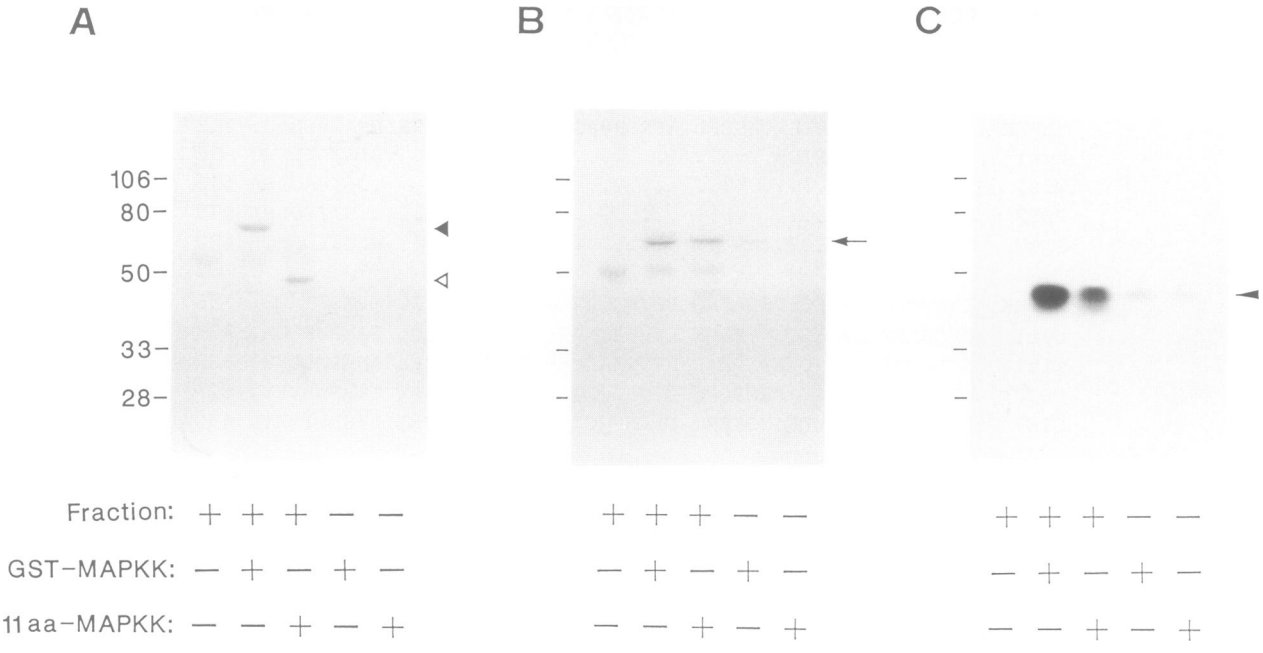

Fig. 6. Phosphorylation (**A**) and activation (**B,C**) of recombinant MAPKK by a fraction of *Xenopus* egg extracts. (A) A Q-Sepharose-adsorbed fraction (Fraction) that was free of *Xenopus* MAPKK was prepared as described in Materials and methods. Recombinant MAPKK (GST−MAPKK or 11aa−MAPKK) was incubated with [γ-^{32}P]ATP in the presence (+) or absence (−) of this fraction. Each sample was electrophoresed and autoradiographed. A closed triangle denotes the position of GST−MAPKK and an open triangle the 11aa−MAPKK. Prestained SDS−PAGE standards (BioRad) were used as molecular weight markers. (B) Recombinant MAPKK (GST−MAPKK or 11aa−MAPKK) had been incubated with (+) or without (−) a Q-Sepharose-adsorbed fraction (Fraction). Phosphorylation of GST−KNMAPK by these recombinant MAPKKs was then examined as described in Materials and methods. An arrow denotes the position of GST−KNMAPK. (C) Recombinant MAPKK which had been incubated with or without a Q-Sepharose-adsorbed fraction was incubated with recombinant MAPK as described in Materials and methods. The MBP-phosphorylating activity of the recombinant MAPK was then measured by the kinase detection assay within MBP-containing gels. An arrowhead denotes the position of recombinant MAPK.

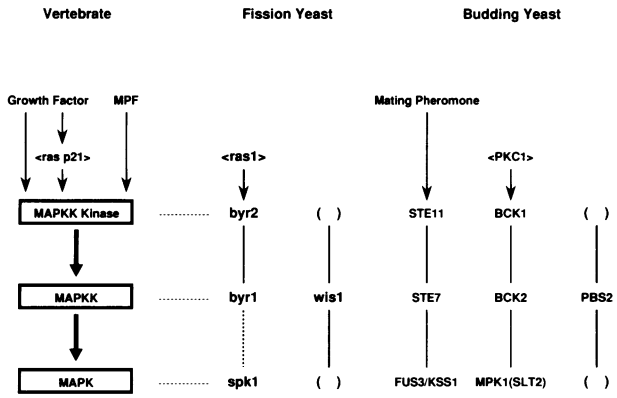

Fig. 7. A hypothetical model for kinase cascades in various signal transduction pathways from yeasts to vertebrates. In vertebrates, MAP kinase (MAPK) is activated by a serine/threonine/tyrosine kinase, MAPKK, which is in turn activated by a serine/threonine kinase, MAPKK kinase. This kinase cascade system may function in the growth factor- or differentiation factor-stimulated signaling pathways and in the MPF-induced signaling pathway. In yeasts, there are several pathways hypothetically analogous to the MAPKK kinase/MAPKK/MAPK cascade: byr2/byr1/spk1, STE11/STE7/FUS3 and/or KSS1, and BCK1/BCK2/SLT2. As wis1 and PBS2, other putative MAPKK homologs, are shown to function in other pathways, there might be other yeast kinases homologous to MAPK and MAPKK kinase. It is also possible that other undiscovered pathways exist. Other details are given in the text.

of 1 mM IPTG for 10 h and then purified by using a GSH−agarose column as described in the purification of GST−MAPKs. Further purification of GST−MAPKK was performed by chromatography on phenyl−Sepharose. The production of MAPKK was confirmed by immunoblotting with affinity-purified anti-MAPKK antibody raised against the N-terminal peptide of *Xenopus* MAPKK (Kosako *et al.*, 1992). A lower band (~35 kDa) detected in Coomassie blue staining may be a degradation product of GST−MAPKK, as it can be seen in the immunoblotting with anti-MAPKK. GST−MAPKK was incubated with 5 μCi [γ-^{32}P]ATP in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$ or 20 mM $MgCl_2$ for 30 min at 37°C. This GST−MAPKK preferred Mn^{2+} to Mg^{2+} as divalent cations for autophosphorylation, like native MAPKK purified from mature oocytes. To remove GST from GST−MAPKK, GST−MAPKK (80 μg) was incubated for 2 h at 30°C with 0.1 mg/ml Factor Xa (DANEX BIOTEK) in a solution consisting of 20 mM Tris−HCl (pH 7.5), 1 mM $CaCl_2$ and 100 μM NaCl in a final volume of 25 μl. The reaction was stopped by the addition of 5 mM dithiothreitol and 2 mM EGTA. This digestion produced an 11aa−MAPKK which contains 11 extra amino acids (GIPGNSALTPN) on the authentic N-terminus of MAPKK.

Phosphorylation and activation of recombinant MAPKK by a fraction of Xenopus egg extracts

A fraction of *Xenopus* egg extracts that contains the activity to phosphorylate and reactivate the phosphatase 2A-treated native MAPKK was prepared as described elsewhere (Matsuda *et al.*, 1993). Briefly, extracts of mature *Xenopus* oocytes were loaded onto a Q-Sepharose column equilibrated with buffer A [20 mM Hepes (pH 7.5), 20 mM β-glycerophosphate, 2 mM EGTA, 10% glycerol, 0.01% Brij35, 20 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM vanadate]. After washing the column with 0.1 M NaCl in buffer A, proteins were eluted with 0.5 M NaCl in buffer A. This fraction, which was free of MAPKK (Matsuda *et al.*, 1993), was concentrated, dialyzed against buffer A and used in the following experiments as Q-Sepharose-adsorbed fraction. To detect the phosphorylation of recombinant MAPKK, GST−MAPKK (0.5 mg/ml) or 11aa−MAPKK (0.15 mg/ml) was incubated for 30 min at 30°C with 8.5 μl of a Q-Sepharose-adsorbed fraction or buffer A in a solution consisting of 20 mM Tris−Cl (pH 7.5), 20 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP (1 μCi) in a final volume of 15 μl. Samples were electrophoresed and autoradiographed. To detect the phosphorylation of GST−KNMAPK by the activated recombinant MAPKK, GST−MAPKK or 11aa−MAPKK was preincubated with a Q-Sepharose-adsorbed fraction as above except that radiolabeled ATP was omitted. Then GST−KNMAPK (0.5 mg/ml) and [γ-^{32}P]ATP (1 μCi) were added, and the incubation was continued for another 30 min. After electrophoresis, the radioactivity of GST−KNMAPK was detected by autoradiography. To detect the activation of recombinant MAPK by the activated recombinant MAPKK, GST−MAPKK or 11aa−MAPKK which had been preincubated with a Q-Sepharose fraction was incubated with recombinant MAPK (50 μg/ml, MAPK with 10 additional amino acids on the authentic N-terminus; Matsuda *et al.*, 1992) for 30 min at 30°C. Subsequently, samples were subjected to the kinase detection assay within MBP-containing gels (Gotoh *et al.*, 1990b).

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Note added in proof

It has recently been reported that phosphorylation of FUS3 and KSS1 on both threonine and tyrosine residues of the TEY sequence depends on STE7 [Gartner *et al.* (1992) *Genes Dev.*, **6**, 1280–1292]. The nucleotide sequence data of MAPKK reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number D13700. Most recently, Krebs *et al.* [(1992) *J. Biol. Chem.*, **267**, 14373–14381] reported the purification and characterization of human 45 and 46 kDa MAPKKs. The protein kinase referred to as BCK2 in the text is now designated as MKK1/MKK2.