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A novel SAPK/JNK kinase, MKK7, stimulated by TNFα and cellular stresses

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Stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), a member of the MAP kinase (MAPK) superfamily, is thought to play a key role in a variety of cellular responses. To date, SEK1/MKK4, one of the MAP kinase kinase (MAPKK) family of molecules, is the only SAPK/JNK kinase that has been cloned. Here we have cloned, identified and characterized a novel member of the mammalian MAPKKs, designated MKK7. MKK7 is most similar to the mediator of morphogenesis, *hemipterous* (*hep*), in *Drosophila*. Immunochemical studies have identified MKK7 as one of the major SAPK/JNK-activating kinases in osmotically shocked cells. While SEK1/MKK4 can activate both the SAPK/JNK and p38 subgroups of the MAPK superfamily, MKK7 is specific for the SAPK/JNK subgroup. MKK7 is activated strongly by tumour necrosis factor α (TNFα) as well as by environmental stresses, whereas SEK1/MKK4 is not activated by TNFα. Column fractionation studies have shown that MKK7 is a major activator for SAPK/JNK in the TNFα-stimulated pathway. Moreover, we have found that overexpression of MKK7 enhances transcription from an AP-1-dependent reporter construct. Thus, MKK7 is an evolutionarily conserved MAPKK isoform which is specific for SAPK/JNK, is involved in AP-1-dependent transcription and may be a crucial mediator of TNFα signalling.

Keywords: MAP kinase/protein kinase cascade/signal transduction/TNFα

Introduction

Mitogen-activated protein kinase (MAPK, also known as ERK) and its direct activator, MAPK kinase (MAPKK, also known as MEK), constitute a functional unit that links a variety of extracellular signals to nuclear events and is essential for cell proliferation, cell differentiation and early embryonic development. MAPK is activated by dual phosphorylation on tyrosine and threonine residues catalysed by MAPKK, which is a dual specificity kinase (Nishida and Gotoh, 1993; Davis, 1994; Cobb and Goldsmith, 1995; Karin and Hunter, 1995; Marshall, 1995; Kyriakis and Avruch, 1996; Treisman, 1996).

Over the past several years it has become apparent that there are two types of MAPK-related kinases, called stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and p38/MPK2/CSBP, both of which are activated in response to a variety of cellular stresses and inflammatory cytokines (Derijard *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). Classical MAPK (ERK), SAPK/JNK and p38 are thought to constitute the MAPK superfamily. Like classical MAPKK (MEK), direct activators for SAPK/JNK and p38 have been identified. Specific activators for p38, MKK3 and MKK6, have been described (Derijard *et al.*, 1995; Han *et al.*, 1996; Moriguchi *et al.*, 1996a; Raingeaud *et al.*, 1996; Stein *et al.*, 1996) and, for SAPK/JNK, SEK1/MKK4 has been identified as a direct activator (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995). However, biochemical studies revealed the existence of other SAPK/JNK activators in cells stimulated by a variety of cellular stresses (Moriguchi *et al.*, 1995; Meier *et al.*, 1996). In addition, recent studies using *sek1*$^{-/-}$ cells indicated that there are SEK1/MKK4-dependent and SEK1/MKK4-independent intracellular signalling pathways for SAPK/JNK activation (Nishina *et al.*, 1997; Yang *et al.*, 1997).

Here we report the cDNA cloning and characterization of a novel member of the mammalian MAPKKs, termed MKK7. MKK7 is most similar to the mediator of morphogenesis, *hemipterous* (*hep*) (Glise *et al.*, 1995), in *Drosophila*. We produced anti-MKK7 antibody and identified MKK7 as one of the major SAPK/JNK-activating kinases in osmotically shocked cells. Moreover, we have shown that while SEK1/MKK4 can activate both the SAPK/JNK and p38 subgroups of the MAPK superfamily, MKK7 specifically activates the SAPK/JNK subgroup. Interestingly, we have found that MKK7 is activated strongly by tumour necrosis factor α (TNFα) as well as by environmental stresses, whereas SEK1/MKK4 is not activated by TNFα. Biochemical studies showed that MKK7 is a major activator for SAPK/JNK in the TNFα-induced signalling pathway. Futhermore, expression of a kinase-negative form of MKK7 was found to interfere with activation of SAPK/JNK induced by TNFα. Thus, SAPK/JNK may be differentially regulated by MKK7 and SEK1/MKK4, and MKK7 may be a crucial mediator of TNFα signalling.

Results and discussion

cDNA cloning and identification of MKK7, a novel MAP kinase kinase

By screening a mouse brain cDNA library, we isolated a cDNA clone which encodes a new member of the MAPKK family, which we designated MKK7. The deduced amino acid sequence indicated that MKK7 is a protein of 468 amino acids, with a predicted M_r of 52.5 kDa. MKK7 is

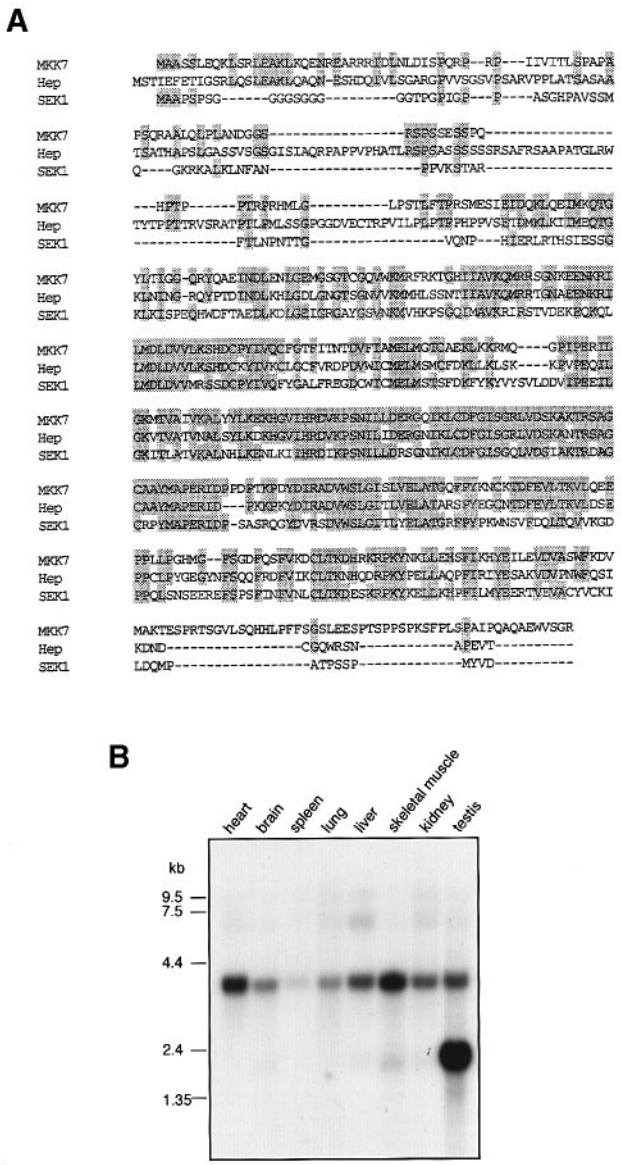


Fig. 1. (**A**) The predicted amino acid sequence of mouse MKK7 was aligned with *Drosophila* Hep (Glise *et al.*, 1995) and mouse SEK1 (Sanchez *et al.*, 1994) amino acid sequences using the Clustal W program. Residues identical to MKK7 are shaded. The sequence of mouse MKK7 has been submitted to DDBJ/EMBL/GenBank under the accession no. AB005654. (**B**) Northern blot analysis, in which MKK7 mRNA was detected by hybridization with a 600 bp DNA probe containing the N-terminal coding sequence. Molecular size markers are shown on the left.

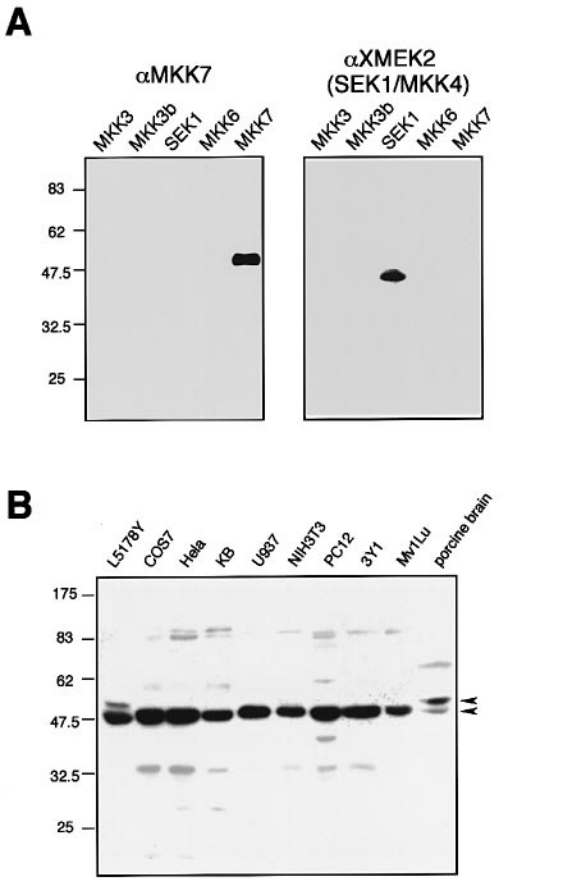


Fig. 2. (**A**) Specificity of anti-MKK7 antibody. COS7 cells were transiently transfected with 2 μg of pSRα-HA-MKK3, pSRα-HA-MKK3b, pSRα-HA-SEK1, pSRα-HA-MKK6 or pSRα-HA-MKK7. Cell extracts were prepared, and equal amounts of proteins (0.5 μg) were subjected to immunoblotting with anti-MKK7 antibody or anti-XMEK2 antibody. (**B**) Detection of endogenous MKK7s. Extracts (10 μg of proteins) from various cells were subjected to immunoblotting with anti-MKK7 antibody. The two major bands that were recognized by anti-MKK7 antibody are indicated by arrowheads.

most similar to SEK1/MKK4 (Sanchez *et al.*, 1994) (53% identity in the kinase domain) of six previously described members of the mammalian MAPKK family. It is, however, closer to a *Drosophila* MAPKK family protein *hemipterous* (Hep) (Glise *et al.*, 1995) (69% identity in the kinase domain) than to SEK1/MKK4 (Figure 1A). Northern blotting revealed that MKK7 was expressed ubiquitously in mouse tissues (Figure 1B). To identify and characterize MKK7 protein, we produced anti-MKK7 antibody by immunizing rabbits with recombinant histidine (His)-tagged MKK7 protein. This antibody reacted specifically with MKK7 protein expressed in COS7 cells, and did not recognize other members of the MAPKK family: MKK3 (Derijard *et al.*, 1995), MKK3b (Moriguchi *et al.*, 1996b), SEK1/MKK4 (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995) or MKK6 (Han *et al.*, 1996; Moriguchi *et al.*, 1996a; Raingeaud *et al.*, 1996; Stein *et al.*, 1996) (Figure 2A, left). On the other hand, the anti-XMEK2 antibody which recognizes SEK1/MKK4 (Moriguchi *et al.*, 1995) did not react with MKK7 (Figure 2A, right). The anti-MKK7 antibody commonly recognized a 48 kDa polypeptide in various mammalian cells, and detected a 52 kDa polypeptide in extracts from mouse L5178Y cells and porcine brain (Figure 2B). The 48 and 52 kDa polypeptides behaved almost identically in various types of chromatographic fractionations (see Figure 3), and the immunodetection of both bands was blocked with recombinant His-MKK7 (data not shown). Thus, the two polypeptides are quite similar in terms of their biochemical and immunological nature. Like other members of the MAPKK family (Cuenda *et al.*, 1996; Han *et al.*, 1996; Moriguchi *et al.*, 1996b), an alternatively spliced form of MKK7 may exist. In fact, we isolated human and *Xenopus* MKK7 cDNA clones encoding a truncated form (C-terminal 33 amino acids deleted) (T.Moriguchi, N.Masuyama and E.Nishida, unpublished data). After submission of this manuscript, a paper appeared reporting

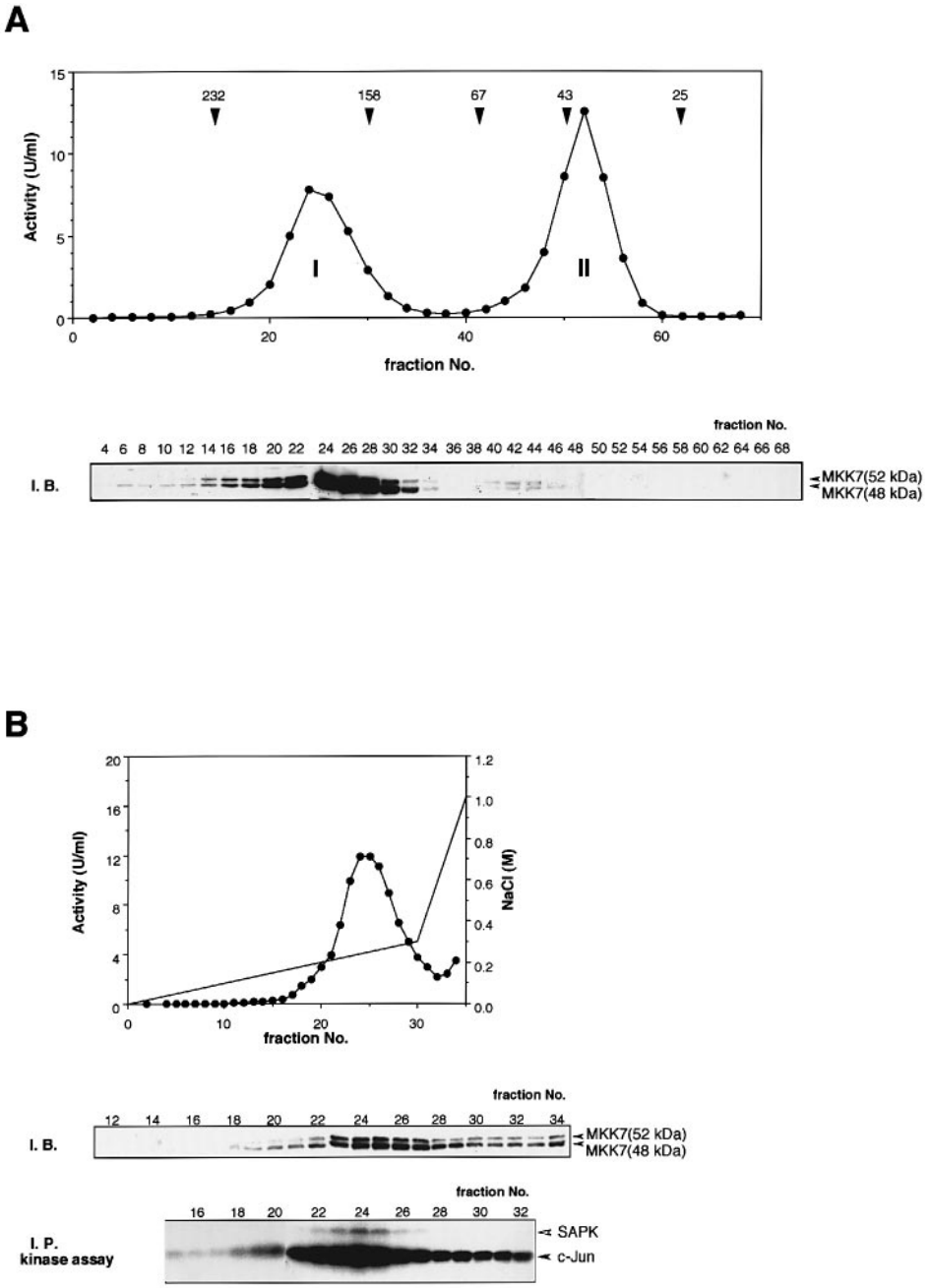
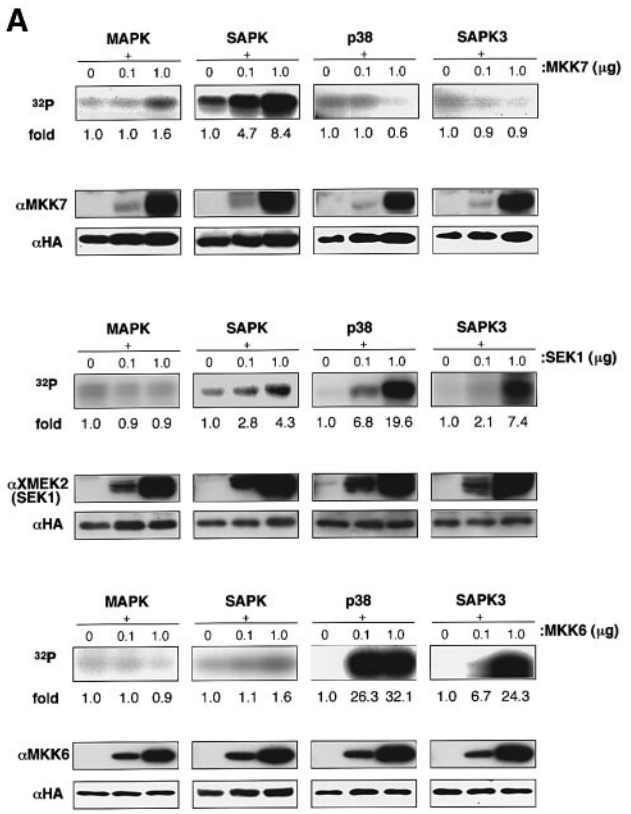


Fig. 3. Identification of MKK7 as one of the major SAPK/JNK activators *in vivo*. (**A**) Chromatography on Superdex-200. Extracts were prepared from 2.0×10^{10} L5178Y cells stimulated with 0.7 M NaCl for 30 min at 37°C, and subjected to sequential chromatography as described (Moriguchi *et al.*, 1995, 1996b). Briefly, SAPK/JNK activators were purified from the extract by batchwise chromatography on Q-Sepharose and blue-Sepharose, and gradient elution from phenyl-Sepharose. A major peak of SAPK/JNK-activating activity on phenyl-Sepharose was concentrated and applied to the HiLoad 16/60 Superdex 200 gel filtration column (Pharmacia Biotech). Each fraction was subjected to kinase assay for SAPK-activating activity and immunoblotting with anti-MKK7 antibody. The positions of the molecular weight markers are indicated by arrowheads in the upper panel. (**B**) Chromatography on heparin–Sepharose. Peak I from the Superdex-200 chromatography was pooled and subjected to chromatography on heparin–Sepharose. Each fraction was subjected to kinase assay for SAPK-activating activity, immunoblotting and immune complex kinase assay using anti-MKK7 antibody.

the cDNA cloning of an alternatively spliced form of mouse MKK7 (Tournier *et al.*, 1997).

MKK7 is one of the major SAPK/JNK activators

Previous studies have suggested that SEK1/MKK4 is not the sole SAPK/JNK activator in mammalian cells, and that other unidentified molecules contribute significantly to the activation of SAPK/JNK in a number of signalling pathways (Moriguchi *et al.*, 1995; Meier *et al.*, 1996). To examine whether MKK7 functions as a major activator for SAPK/JNK *in vivo*, we performed immunoblotting and immune complex kinase assays in extracts obtained from cells exposed to osmotic stress. By fractionating the extracts from L5178Y cells exposed to hyperosmolarity, we showed that 15–20% of the SAPK/JNK-activating activity adsorbed to Q-Sepharose and the remainder (80–85% of the activity) eluted unadsorbed. The former was shown to be MKK4/SEK1 (Moriguchi *et al.*, 1995).



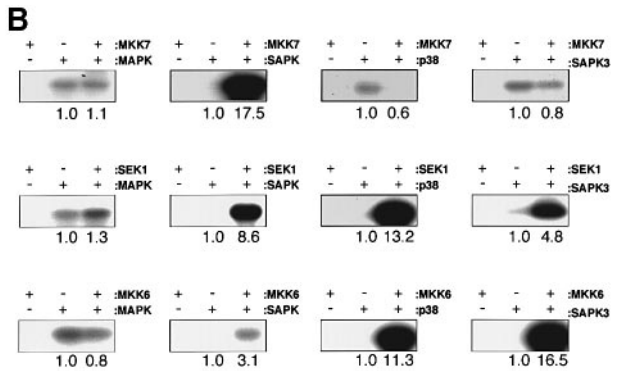


Fig. 4. MKK7 is specific for the SAPK/JNK subgroup. (**A**) Specific activation of SAPK upon co-expression with MKK7. The indicated amounts of pSRα-MKK7, pSRα-SEK1 or pSRα-MKK6 were transfected into COS7 cells with 1 μg of pSRα-HA-MAPK, pSRα-HA-SAPKα, pSRα-HA-p38 or pSRα-HA-SAPK3. The empty pSRα vector was used to achieve equal amounts of each vector in each transfection. The expression levels of MKK7, SEK1 or MKK6 were monitored by immunoblotting of cell lysates [αMKK7, αXMEK2(SEK1) or αMKK6]. The kinase activity of HA-tagged MAPK, SAPKα, p38 or SAPK3 was measured by immune complex kinase assay using their respective substrates [myelin basic protein for MAPK and SAPK3, GST-cJun(1–79) for SAPKα or ATF-2 for p38]. The amount of HA-MAPKs in each immune complex was determined by immunoblotting (αHA). The fold increase in the activity of HA-tagged MAPKs was quantified by image analyser (Fujix BAS2000). Similar results were obtained in three different experiments. (**B**) MKK7 activates SAPK *in vitro*. His-MKK7, GST-SEK1 or His-MKK6 were incubated with His-MAPK, His-SAPKα, His-p38 or His-SAPK3 in the presence of ATP and Mg^{2+}. The activity of MAPK, SAPKα, p38 or SAPK3 was then measured using the substrates described above. Similar results were obtained in two different experiments.

The latter fraction was purified further by sequential chromatography on blue-Sepharose and phenyl-Sepharose, and then the broad activity peak was subjected to gel filtration on Superdex-200. Two peaks of SAPK/JNK-activating activity were observed; one (peak I) eluted at the position corresponding to an apparent M_r of 180 kDa, and the other (peak II) at an apparent M_r of 40 kDa (Figure 3A, upper). MKK7 proteins (both the 48 and 52 kDa polypeptides) co-eluted with peak I (Figure 3A, fraction 20–30), and a trace of MKK7 eluted at the position of an apparent M_r of 55 kDa (Figure 3A, fraction 42–44), distinct from peak II (fraction 48–54). Peak I and peak II were subjected separately to chromatography on heparin–Sepharose. The SAPK/JNK-activating activity from peak I was eluted at 0.25 M NaCl, completely coincident with the elution of MKK7 proteins (Figure 3B, upper and middle). The SAPK/JNK-activating activity of the immunoprecipitate with anti-MKK7 antibody from each fraction also coincided with the total activity of each fraction (Figure 3B, lower cf. upper). On the other hand, the SAPK/JNK-activating activity from peak II was eluted at 0.18 M NaCl on heparin–Sepharose (data not shown). No MKK7 polypeptide was observed on immunoblotting, and no activity was detected in the immunoprecipitate with anti-MKK7 antibody (data not shown). From these data, it is concluded that peak I is MKK7 and peak II is a hitherto unidentified molecule. Thus, MKK7 is one of the major SAPK/JNK activators in the osmotic stress-induced pathway, and it may exist as a non-monomeric form. Whether it forms a homo-oligomer or complexes with other proteins remains to be elucidated.

MKK7 specifically activates SAPK/JNK

SEK1/MKK4, the previously known SAPK/JNK activator, has been shown to activate not only SAPK/JNK but also p38 (Derijard *et al.*, 1995; Lin *et al.*, 1995), another subgroup of the MAPK superfamily. To identify the substrate specificity of MKK7, we tested the activity of MKK7 toward four distinct classes of the MAPK superfamily, classical MAPK (here simply called MAPK, also known as ERK), SAPK(/JNK), p38 and a recently reported molecule, SAPK3/ERK6 (Lechner *et al.*, 1996; Mertens *et al.*, 1996). SAPK3(/ERK6) is structurally similar to SAPK/JNK or p38, and has the Thr–Gly–Tyr sequence in the activation phosphorylation site, like p38. Increasing amounts of a plasmid expressing MKK7, SEK1(/MKK4) or MKK6 were transfected into COS7 cells together with either of the plasmids expressing haemagglutinin (HA)-tagged MAPK superfamily molecules, the kinase activity of HA-tagged protein was assayed with each substrate and the immunoprecipitated protein was quantified by immunoblotting with anti-HA antibody. Expression of MKK7 led to specific activation of SAPK/JNK (Figure 4A). In contrast, expression of SEK1 resulted in activation of both SAPK/JNK and p38 and also activation of SAPK3 (Figure 4A). MKK6 could activate p38 and SAPK3 strongly and could not activate SAPK/JNK or MAPK (Figure 4A).

Next, the substrate specificity of these MAPKK family molecules was examined *in vitro*. Each of the MAPKK molecules was bacterially produced and their activity toward bacterially produced MAPK family molecules was assayed. This *in vitro* kinase assay gave results essentially

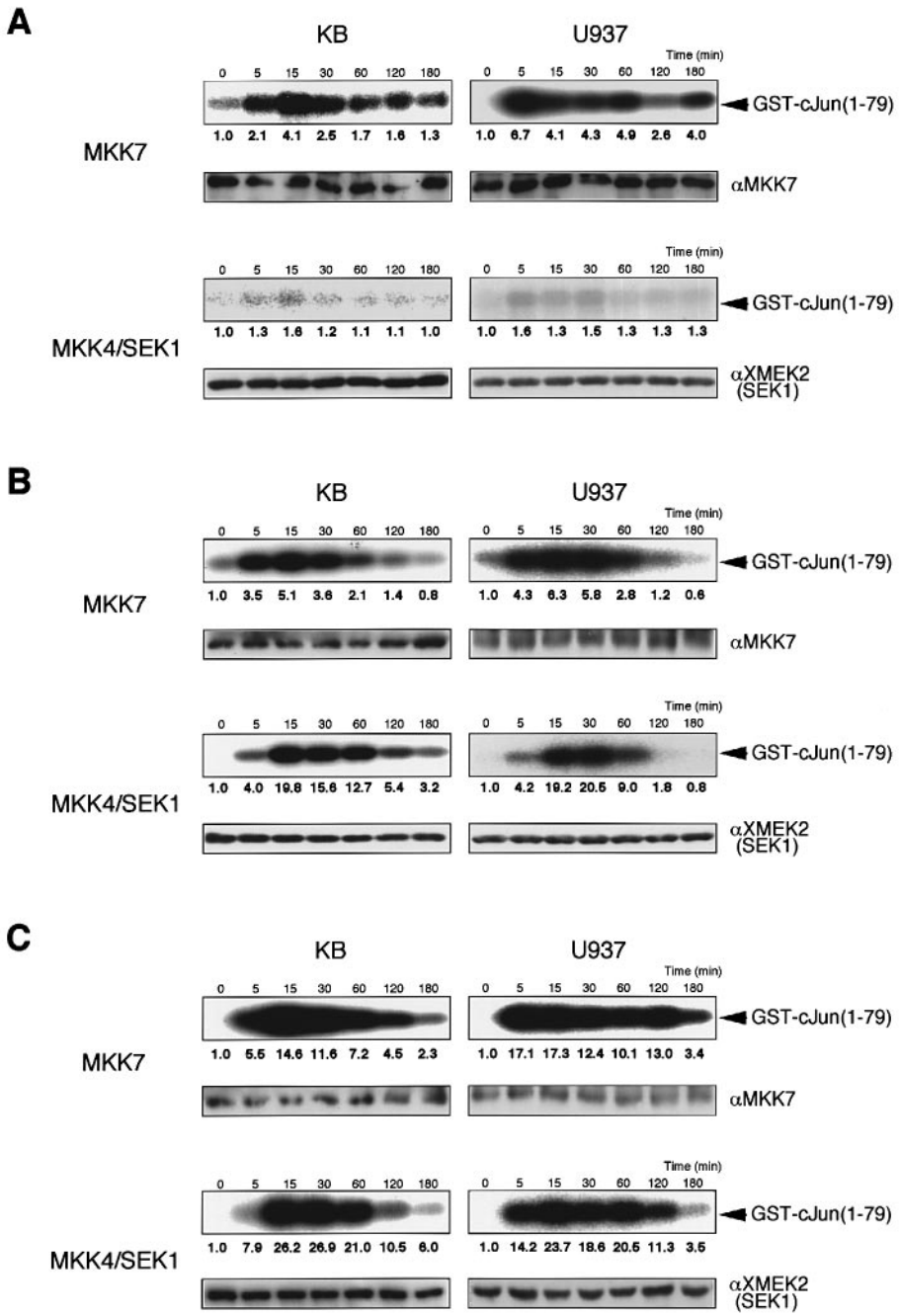


Fig. 5. Differential activation of endogenous MKK7 and endogenous SEK1/MKK4 by various stimuli. KB cells or U937 cells were incubated with 100 ng/ml TNFα (for KB), 20 ng/ml TNFα (for U937) (**A**), 100 μg/ml anisomycin (**B**) or 0.5 M sorbitol (**C**) for the indicated times. The kinase activity of endogenous MKK7 or endogenous SEK1/MKK4 for activating SAPK was measured by the immune complex kinase assay using His-SAPK and GST-cJun(1–79) as substrates. The amount of MKK7 or SEK1/MKK4 in each immune complex was determined by immunoblotting (αMKK7 or αXMEK2). Phosphorylation of GST-cJun(1–79) was quantified by image analyser (Fujix BAS2000). Similar results were obtained in at least four different experiments.

identical to those obtained from the co-transfection assay (Figure 4B). From these two assays, we can conclude that while SEK1/MKK4 can activate both the SAPK/JNK and p38 subgroups of the MAPK superfamily, MKK7 is a specific activator for SAPK/JNK (see Figure 8) and that MKK6 is a specific activator for the p38 subgroup including SAPK3 (see Figure 8). It should be noted, however, that our assays measure the specificities seen *in vitro* and with proteins overexpressed in COS7 cells, and might not necessarily reflect the specificities *in vivo*.

MKK7 and SEK1/MKK4 are differentially activated by TNFα and cellular stresses

To examine whether MKK7 and SEK1/MKK4, two SAPK/JNK activators, have different response characteristics, we compared their activation kinetics in response to various stimuli. TNFα, one of the major activating stimuli for SAPK/JNK (Sluss *et al.*, 1994), elicits a wide variety of cellular responses including gene expression, cell proliferation, cell differentiation and cell death (Vandenabeele *et al.*, 1995). The two cell lines (KB cells and U937 cells)

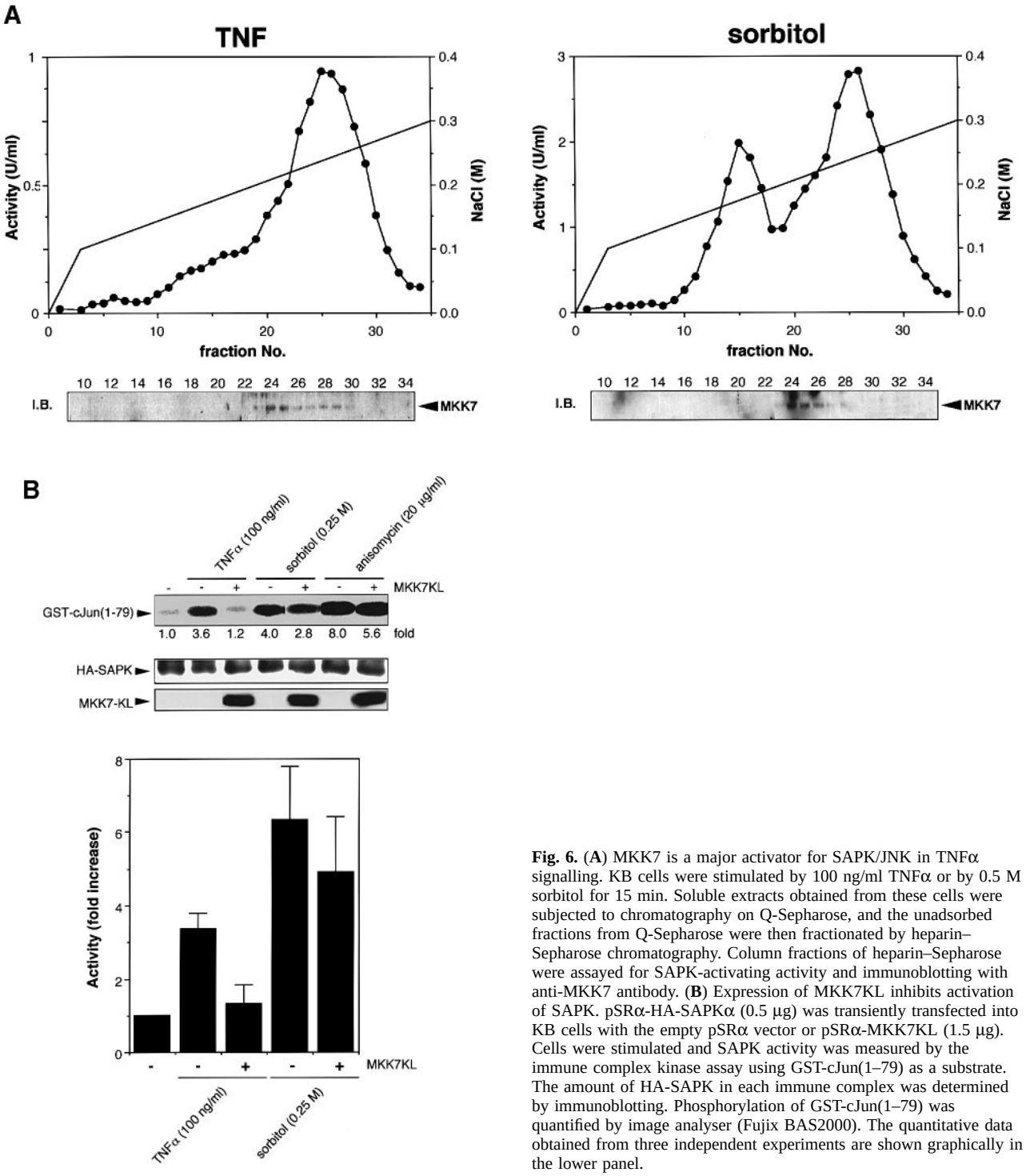


Fig. 6. (**A**) MKK7 is a major activator for SAPK/JNK in TNFα signalling. KB cells were stimulated by 100 ng/ml TNFα or by 0.5 M sorbitol for 15 min. Soluble extracts obtained from these cells were subjected to chromatography on Q-Sepharose, and the unadsorbed fractions from Q-Sepharose were then fractionated by heparin–Sepharose chromatography. Column fractions of heparin–Sepharose were assayed for SAPK-activating activity and immunoblotting with anti-MKK7 antibody. (**B**) Expression of MKK7KL inhibits activation of SAPK. pSRα-HA-SAPKα (0.5 μg) was transiently transfected into KB cells with the empty pSRα vector or pSRα-MKK7KL (1.5 μg). Cells were stimulated and SAPK activity was measured by the immune complex kinase assay using GST-cJun(1–79) as a substrate. The amount of HA-SAPK in each immune complex was determined by immunoblotting. Phosphorylation of GST-cJun(1–79) was quantified by image analyser (Fujix BAS2000). The quantitative data obtained from three independent experiments are shown graphically in the lower panel.

expressing both MKK7 and SEK1/MKK4 endogenously were exposed to TNFα. MKK7 was activated rapidly and strongly by TNFα in both cell lines, whereas SEK1/MKK4 was not activated significantly (Figure 5A). When these cells were exposed to environmental stresses, such as the protein synthesis inhibitor anisomycin (Figure 5B) or hyperosmolarity (Figure 5C), both MKK7 and SEK1/MKK4 were activated strongly. Interestingly, the activation kinetics of the two kinases differed from each other; MKK7 was activated more rapidly than SEK1/MKK4 in both pathways (Figure 5B and C). These results suggest that MKK7 and MKK4/SEK1, two major activators for SAPK/JNK, have different upstream activating pathways.

MKK7 is a major activator for SAPK/JNK in the TNFα signalling pathway

To clarify further the role of MKK7 in TNFα signalling, we performed fractionation studies. The SAPK/JNK-activating activity was not detected in the adsorbed fraction in Q-Sepharose chromatography of the TNFα-stimulated cell extracts. The unadsorbed fractions were then subjected to heparin–Sepharose chromatography. The SAPK/JNK-

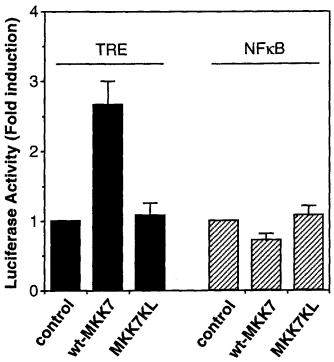


Fig. 7. MKK7 is involved in AP-1-dependent transcription. KB cells were transfected with the wild-type MKK7 plasmid (wt-MKK7), the MKK7KL plasmid or control plasmid. The reporter plasmid, pTREx3-tk-Luc (for transcription from the AP-1-dependent reporter) (Tanaka *et al.*, 1994) or p55IgκLuc (for transcription from the NF-κB-dependent reporter) (Fujita *et al.*, 1993), was co-transfected with each expression plasmid. After 48 h, the cells were lysed and assayed for luciferase activity. The results are averages of three separate experiments.

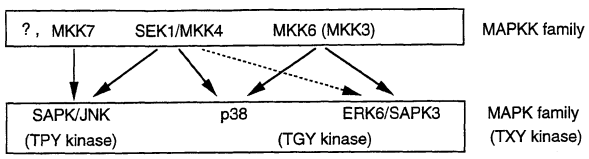


Fig. 8. Schematic representation of MAPK superfamily signal transduction pathways.

activating activity eluted mainly at 0.25 M NaCl on heparin–Sepharose, coincident with the elution of MKK7 proteins (Figure 6A, TNF). On the other hand, the SAPK/JNK-activating activity from sorbitol-treated KB cells was resolved into two peaks on heparin–Sepharose (Figure 6A, sorbitol). The activity peak at 0.18 M NaCl may correspond to the third SAPK/JNK activator (see Figure 3A and data not shown). These results demonstrate that MKK7 is a major SAPK/JNK activator in the TNFα signalling pathway. We then examined the effect of expression of a kinase-negative form of MKK7 (MKK7KL) on SAPK/JNK activity in response to extracellular stimuli. The TNFα-induced activation of SAPK/JNK was almost completely suppressed, while the hyperosmolarity- or anisomycin-induced activation was partially suppressed (Figure 6B). These results are consistent with the results of the fractionation studies described above.

MKK7 is involved in AP-1-dependent transcription

It is known that TNFα induces activation of two transcription factors, AP-1 (Brenner *et al.*, 1989) and NF-κB (Osborn *et al.*, 1989). To address the possible role of MKK7 in these transcriptional activities, we examined the effect of expression of wild-type MKK7 or a kinase-dead form of MKK7(MKK7KL) on the transcription from an AP-1-dependent reporter construct or that from an NF-κB-dependent reporter construct. Overexpression of MKK7, but not that of MKK7KL, enhanced transcription from an AP-1-dependent reporter gene (Figure 7, TRE), but not from an NF-κB-dependent reporter gene (Figure 7, NF-κB). This may be in agreement with the recent observations that the SAPK/JNK activation is not linked to NF-κB activation (Liu *et al.*, 1996; Reinhard *et al.*, 1997).

Conclusions

Three subgroups of the MAPK superfamily, classical MAPK (also called ERK), SAPK/JNK and p38, are distinguished by the sequence of the tripeptide dual phosphorylation motif (Nishida and Gotoh, 1993; Davis, 1994; Cobb and Goldsmith, 1995; Karin and Hunter, 1995; Marshall, 1995; Kyriakis and Avruch, 1996; Treisman, 1996). A direct and specific activator for classical MAPK has been identified as classical MAPKK (also known as MEK) (Nishida and Gotoh, 1993; Cobb and Goldsmith, 1995; Marshall, 1995). Also, specific activators for p38 previously have been identified as MKK3 and MKK6 (Derijard *et al.*, 1995; Han *et al.*, 1996; Moriguchi *et al.*, 1996a; Raingeaud *et al.*, 1996; Stein *et al.*, 1996). SEK1/MKK4 is the only molecule that has been identified previously as an activator for SAPK/JNK (Derijard *et al.*, 1995; Lin *et al.*, 1995) and has been reported to be able to activate both SAPK/JNK and p38 *in vitro* and when overexpressed in COS cells. Here MKK7 has been shown to be a specific activator for SAPK/JNK, which functions in TNFα-induced or environmental stress-induced signalling pathways as an *in vivo* activator for SAPK/JNK. Moreover, MKK7 is a major activator for SAPK/JNK in TNFα-stimulated KB cells, and a kinase-negative MKK7 is able to suppress TNFα-mediated SAPK/JNK activation in KB cells. Thus, MKK7 constitutes an essential part of TNFα-induced signal transductions. MKK7 is most similar to the mediator of morphogenesis, *hemipterous* (*hep*), in *Drosophila* (Glise *et al.*, 1995). *hep* is required in the embryo for dorsal closure, a process involving coordinate cell shape changes of ectodermal cells. Recently, it was reported that the *Drosophila* homologue of SAPK/JNK, DJNK, is encoded by *basket*, which is also required for dorsal closure (Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996). Further, DJNK can be activated by the *Drosophila* MAPKK family molecule Hep *in vitro* (Sluss *et al.*, 1996). Thus, Hep and DJNK may function in the same signal transduction pathway in *Drosophila*. Most recently, a *Drosophila* homologue of SEK1/MKK4, distinct from Hep/MKK7, was isolated and shown to be able to activate both DJNK and a *Drosophila* homologue of p38 *in vitro* (K.Irie and K.Matsumoto, personal communication). Therefore, both in vertebrate and in *Drosophila* there are at least two SAPK/JNK activators, SEK1/MKK4 and MKK7/Hep. The former can activate both the SAPK/JNK and p38 subgroups of the MAPK superfamily, whereas the latter is specific for SAPK/JNK. Thus, this study, by identifying a novel, specific activator for SAPK/JNK, defines an evolutionarily conserved signalling module.

Materials and methods

Molecular cloning

A *Xenopus* oocyte cDNA library (Clontech) was screened at reduced stringency (final wash at 42°C in 2× SSC, 0.1% SDS) using the human MKK3 coding region as a probe. One of the positive clones encodes a novel kinase that is similar to *Drosophila hep*, but it represents a partial transcript (N.Masuyama, T.Moriguchi and E.Nishida, unpublished data). Using this clone as a probe, a mouse brain cDNA library (Stratagene) was hybridized at reduced stringency (see above). A mouse clone (named MKK7) was isolated. For Northern analysis, mouse multiple tissue Northern blot (Clontech) was used.

Mutagenesis

The mutagenic oligonucleotide (CAGGCCACATCATTGCTGTTCTGC-AGATGCGGCGCTCTGGGAAC) was used to convert Lys165 of MKK7 to Leu. The mutation was confirmed by DNA sequencing.

Cell culture

U937 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum. KB cells were maintained in DMEM supplemented with 10% bovine serum. COS7 cells were cultured as described previously (Moriguchi *et al.*, 1996a).

Transient transfection

The MKK7-coding region was subcloned into pcDL-SRα456 or pSRα-HA1 (Moriguchi *et al.*, 1996a). The rat SAPK3-coding region was amplified by PCR using rat skeletal muscle cDNA (Clontech) as a template and subcloned into pSRα-HA1. Other plasmids used here have been described elsewhere (Moriguchi *et al.*, 1996a,b). COS7 cells or KB cells were transiently transfected by Lipofectamine (Gibco-BRL) using the amounts of DNA indicated. After 24 h, cell lysates were prepared as described (Moriguchi *et al.*, 1996a) and subjected to immunoblotting or immune kinase assays.

Immunoblotting

MKK7 cDNA was inserted into pET28a (Novagen) to obtain His-tagged protein. Anti-MKK7 polyclonal antibodies were raised in rabbits by immunizing them with His-tagged MKK7. Immunoblotting for MKK7 was performed with a 1:1000 dilution of anti-MKK7 antibody and detected with horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody using Renaissance (DuPont-NEN). Immunoblotting with anti-XMEK2 antibody or anti-HA antibody was done as described (Moriguchi *et al.*, 1995, 1996a).

Kinase assays

The SAPK/JNK-activating activity of the column fractions was assayed by their ability to activate His-SAPK, as described (Moriguchi *et al.*, 1996b). Control experiments were carried out in which His-SAPK was omitted. One unit of SAPK/JNK activity was the amount of enzyme which incorporated 1 nmol of phosphate into c-Jun per minute. One unit of SAPK/JNK-activating activity was the amount which increased the SAPK/JNK activity by 1 U/min. Immunoprecipitation of HA epitope-tagged proteins or SEK1/MKK4 was performed as described (Moriguchi *et al.*, 1995, 1996a). For immunoprecipitation of MKK7, cell extracts (200 μl) were incubated with 3 μl of anti-MKK7 antibody. Immune complex kinase reactions of HA-MAPK, HA-SAPKα, HA-p38 and HA-SAPK3 were performed in a final volume of 15 μl containing 20 mM Tris–HCl (pH 7.5), 2 mM EGTA, 15 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP and 3 μg of myelin basic protein, GST-tagged amino-terminal c-Jun fragment(1–79) or activating transcription factor 2 (ATF-2). For detection of MKK7 or SEK1/MKK4 kinase activities, 0.5 μg of His-SAPKα and 3 μg of GST-cJun(1–79) were used as substrates. Samples were incubated at 30°C for 20 min. Reactions were terminated by the addition of sample buffer and boiling. Substrate phosphorylation was detected and quantified by autoradiography and image analyser (Fujix BAS2000). *In vitro* kinase assays were performed using 0.1 μg of MAPKKs (His-MKK7, GST-SEK1/MKK4 and His-MKK6), 0.5 μg of MAPKs (His-MAPK, His-SAPKα, His-p38 and His-SAPK3) and 3 μg of MAPKs' substrates [myelin basic protein, GST-cJun(1–79) and ATF-2]. GST-SEK1, GST-cJun(1–79) and His-SAPK3 were prepared using pGEX-2T, pGEX-3X (Pharmacia Biotech) and pET28a (Novagen), respectively. Other recombinant proteins have been described elsewhere (Moriguchi *et al.*, 1995, 1996a,b).

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References

Brenner,D.A., O'Hara,M., Angel,P., Chojkier,M. and Karin,M. (1989) Prolonged activation of jun and collagenase genes by tumour necrosis factor-alpha. *Nature*, **337**, 661–663.

Cobb,M.H. and Goldsmith,E.J. (1995) How MAP kinases are regulated. *J. Biol. Chem.*, **270**, 14843–14846.

Cuenda,A., Alonso,G., Morrice,N., Jones,M., Meier,R., Cohen,P. and Nebreda,A.R. (1996) Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine-stimulated monocytes and epithelial cells. *EMBO J.*, **15**, 4156–4164.

Davis,R.J. (1994) MAPKs: new JNK expands the group. *Trends Biochem. Sci.*, **19**, 470–473.

Derijard,B., Hibi,M., Wu,I.H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.

Derijard,B., Raingeaud,J., Barrett,T., Wu,I.H., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, **267**, 682–685.

Fujita,T., Nolan,G.P., Liou,H.-C., Scott,M.L. and Baltimore,D. (1993) The candidate proto-oncogene *bcl-3* encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. *Genes Dev.*, **7**, 1354–1363.

Glise,B., Bourbon,H. and Noseli,S. (1995) *hemipterous* encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. *Cell*, **83**, 451–462.

Han,J., Lee,J.D., Bibbs,L. and Ulevitch,R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265**, 808–811.

Han,J., Lee,J.D., Jiang,Y., Li,Z., Feng,L. and Ulevitch,R.J. (1996) Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.*, **271**, 2886–2891.

Karin,M. and Hunter,T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.*, **5**, 747–757.

Kyriakis,J.M. and Avruch,J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *Bioessays*, **18**, 567–577.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160.

Lechner,C., Zahalka,M.A., Giot,J.F., Moller,N.P. and Ullrich,A. (1996) ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc. Natl Acad. Sci. USA*, **93**, 4355–4359.

Lee,J.C. *et al.* (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.

Lin,A., Minden,A., Martinetto,H., Claret,F.X., Lange-Carter,C., Mercurio,F., Johnson,G.L. and Karin,M. (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. *Science*, **268**, 286–290.

Liu,Z.-G., Hsu,H., Goeddel,D.V. and Karin,M. (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell*, **87**, 565–576.

Marshall,C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.

Meier,R., Rouse,J., Cuenda,A., Nebreda,A.R. and Cohen,P. (1996) Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. *Eur. J. Biochem.*, **236**, 796–805.

Mertens,S., Craxton,M. and Goedert,M. (1996) SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases. *FEBS Lett.*, **383**, 273–276.

Moriguchi,T., Kawasaki,H., Matsuda,S., Gotoh,Y. and Nishida,E. (1995) Evidence for multiple activators for stress-activated protein kinase/c-Jun amino-terminal kinases. Existence of novel activators. *J. Biol. Chem.*, **270**, 12969–12972.

Moriguchi,T. *et al.* (1996a) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.*, **271**, 13675–13679.

Moriguchi,T. *et al.* (1996b) Purification and identification of a major activator for p38 from osmotically shocked cells. Activation of mitogen-activated protein kinase kinase 6 by osmotic shock, tumor necrosis factor-alpha, and H_2O_2. *J. Biol. Chem.*, **271**, 26981–26988.

Nishida,E. and Gotoh,Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.*, **18**, 128–131.

Nishina,H. *et al.* (1997) Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature*, **385**, 350–353.

Osborn,L., Kunkel,S. and Nabel,G.J. (1989) Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. *Proc. Natl Acad. Sci. USA*, **86**, 2336–2340.

Raingeaud,J., Whitmarsh,A.J., Barrett,T., Derijard,B. and Davis,R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255.

Reinhard,C., Shamoon,B., Shyamala,V. and Williams,L.T. (1997) Tumor necrosis factor α-induced activation of c-jun N-terminal kinase is mediated by TRAF2. *EMBO J.*, **16**, 1080–1092.

Riesgo-Escovar,J.R., Jenni,M., Fritz,A. and Hafen,E. (1996) The *Drosophila* Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes Dev.*, **10**, 2759–2768.

Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.

Sanchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature*, **372**, 794–798.

Sluss,H.K., Barrett,T., Derijard,B. and Davis,R.J. (1994) Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol.*, **14**, 8376–8384.

Sluss,H.K., Han,Z., Barrett,T., Davis,R.J. and Ip,Y.T. (1996) A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. *Genes Dev.*, **10**, 2745–2758.

Stein,B., Brady,H., Yang,M.X., Young,D.B. and Barbosa,M.S. (1996) Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. *J. Biol. Chem.*, **271**, 11427–11433.

Tanaka,T., Nishida,J., Mitani,K., Ogawa,S., Yazaki,Y. and Hirai,H. (1994) Evi-1 raises AP-1 activity and stimulates c-fos promoter transactivation with dependence on the second zinc finger domain. *J. Biol. Chem.*, **269**, 24020–24026.

Tournier,C., Whitmarsh,A.J., Cavanagh,J., Barrett,T. and Davis,R.J. (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH_2-terminal kinase. *Proc. Natl Acad. Sci. USA*, **94**, 7337–7342.

Treisman,R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.*, **8**, 205–215.

Vandenabeele,P., Declercq,W., Beyaert,R. and Fiers,W. (1995) Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol.*, **5**, 392–399.

Yang,D., Tournier,C., Wysk,M., Lu,H.-T., Xu,J., Davis,R.J. and Flavell,R.A. (1997) Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH_2-terminal kinase activation, and defects in AP-1 transcriptional activity. *Proc. Natl Acad. Sci. USA*, **94**, 3004–3009.

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