Activation of Stress-activated Protein Kinases/c-Jun N-terminal Protein Kinases (SAPKs/JNKs) by a Novel Mitogen-activated Protein Kinase Kinase (MKK7)*

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Mitogen-activated protein kinase (MAPK) kinases (MKKs) are dual-specificity protein kinases that phosphorylate and activate MAPK. We have isolated a cDNA encoding a novel protein kinase that has significant homology to MKKs. The novel kinase MKK7 has a nucleotide sequence that encodes an open reading frame of 347 amino acids with 11 kinase subdomains. MKK7 is ubiquitously expressed in all adult and embryonic organs but displays high expression in epithelial tissues at later stages of fetal development. When transiently expressed in 293 cells, MKK7 specifically activated stress-activated protein kinases (SAPKs)/c-Jun N-terminal protein kinases (JNKs) but not extracellular-regulated kinase or p38 kinase. A kinase-negative mutant of MKK7 inhibits interleukin-1β, lipopolysaccharide, and MEKK1-induced SAPK/JNK activation. Thus, MKK7 is a new member of the MAPK kinase family that functions upstream of SAPK/JNK in the SAPK/JNK signaling pathway.

Mitogen-activated protein kinases (MAPKs)1 are a family of serine/threonine protein kinases that include extracellular signal-regulated kinases ERK1 and ERK2 (1), stress-activated protein kinases (SAPKs) or c-Jun N-terminal kinases (JNKs) (2–4), p38 kinase (5), and ERK5 (6, 7). MAPKs transduce signals from the cell membrane to the nucleus in response to a variety of different stimuli and participate in various intracellular signaling pathways that control a wide spectrum of cellular processes including cell growth, differentiation, and stress responses (8–10). In contrast to ERK1 and ERK2, which are activated by mitogenic stimuli from growth factor receptors, SAPKs/JNKs are activated in response to stress-inducing signals such as osmotic and heat shock, UV- and γ-irradiation, protein synthesis inhibitors, metabolic poisons, lipopolysaccharide (LPS), or proinflammatory cytokines such as interleukin (IL) 1β (2–4). Once activated, SAPKs/JNKs phosphorylate several transcription factors including c-Jun (3), ATF-2 (12), and ELK-1 (13), thereby regulating gene expression.

The phosphorylation cascades of the MAPK pathways consist of MAPK kinase kinases (MAPKKKs or MEKKs), MAPK kinases (MKKs), and MAPK (8). MEKK phosphorylates and activates a dual-specificity protein kinase, MKK, which in turn phosphorylates MAPK (8). MAPKs are activated through phosphorylation on both threonine and tyrosine residues at the Thr-Tyr dual-phosphorylation motif (14–16). Several mammalian MKKs have been identified to date that include MEK1, MEK2, MKK3, MKK4 (also known as stress-activated protein kinase kinase 1 (SEK1) or JNKK), MEK5, and MKK6. MEK1 and MEK2 catalyze the phosphorylation of ERK1/2 (17, 18), MKK3 and MKK6 specifically activate p38 (19, 20), and MKK4/SEK1 mediates the activation of SAPKs/JNKs (21, 22). MEK5 associates with ERK5, but direct phosphorylation and activation of ERK5 by MEK5 have not been demonstrated (6).

Studies using chromatographically fractionated extracts from several different cell lines suggested that multiple SAPK/JNK activators may exist in addition to MKK4/SEK1 (23, 24). Moreover, recent genetic studies in MKK4/SEK1 gene-deficient murine embryonic stem cells have demonstrated that MKK4/SEK1 is the critical activator of SAPKs/JNKs in response to the protein synthesis inhibitor anisomycin and heat shock, whereas SAPK/JNK activity was still inducible in SEK1-deficient cells after osmolarity changes or UV irradiation (25, 26). The second activator of SAPKs/JNKs operates independently from MKK4/SEK1 and defines a distinct signal transduction pathway, indicating that different stresses utilize distinct signaling pathways for SAPK/JNK activation (25). In addition, phorbol 12-myristate 13-acetate/Ca2+ ionophore stimulation induced SAPK/JNK activation in mature T cells but not in immature thymocytes from SEK1−/− mice, implying that signaling pathways for SAPK activation are developmentally regulated during lymphopoiesis (27). Only MKK4/SEK1 has been molecularly cloned (21, 22).

Although MKK4/SEK1 is structurally related to other MKKs, none of the other MKKs identified so far can activate SAPKs/JNKs (28). We report the isolation of a novel murine protein kinase, MKK7, that specifically activates the SAPKs/JNKs in the SAPK/JNK signaling pathway.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) AF026216.

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§ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MKK or MEK, MAPK kinase; MKKX, MEK kinase; SEK, stress-activated protein kinase kinase; EST, expressed sequence tag; GST, glutathione S-transferase; Ab, antibody; mAb, monoclonal Ab; HA, hemagglutinin; IL-1β, interleukin-1β; LPS, lipopolysaccharide; E, embryonic day.
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JNK pathway but not ERKs or p38 kinases. MKK7 is ubiquitously expressed in adult and embryonic tissues and displays high sequence homology with mammalian MKks and the Drosophila hemipterous (hep) gene (29). Furthermore, a kinase-negative mutant of MKK7-inhibited IL-1β, LPS, and MEKK1-induced SAPK/JNK activation.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Transfections—Recombinant GST-c-Jun (1–169) protein was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant PHAS-I protein was purchased from Stratagene (La Jolla, CA). The phospho-specific SAPK/JNK antibody (Ab) and SAPK/JNK Ab were purchased from New England Biolabs (Beverly, MA). ATP-2 was prepared as described.2 Hemagglutinin (HA) epitope-tagged p54SAPKβ in pMT vector, HA-tagged p38 in pcDNA3 vector, HA-tagged ERK2 in pcDNA3 vector, and MEKK1 in pcDNA have been described previously (31–32).2 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin. DNA (4.0 μg) was transfected into 293 cells as described (31, 32).

cDNA Library Screening, Plasmid Construction and Northern Blot Analysis—A mouse-expressed sequence tag (EST) (444 base pairs) with homology to MKK4 and MKK3 was identified in the Amgen EST database described previously (31–32).2 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin. DNA (4.0 μg) was transfected into 293 cells as described (31, 32).

To identify novel MKks, we searched the Amgen EST data base with MKK4 nucleotide sequences as query sequences. One partial mouse cDNA sequence (444 base pairs) with homology to MKK4 was identified. This cDNA fragment was subsequently used to screen a mouse spleen cDNA library, and several cDNA clones were isolated. Sequencing of the longer clone (3.4 kilobases) showed that it contained a single open reading frame of 347 amino acids, with a predicted molecular mass of 39 kDa. The nucleotide and deduced amino acid sequences of MKK7 are shown in Fig. 1A. There are in-frame stop codons at the 5′ end. The deduced amino acid sequence predicts a kinase catalytic domain at the N terminus and contains 11 kinase subdomains. Comparison of this clone with GenBank™ or EBI data bases found the MKK7 sequence to be novel. However, it showed significant homology to the Drosophila melanogaster Oregon R MAPK kinase hemipterous (hep) (29), mouse and human SEK1/MKK4 (21), MKK6 (20), MKK3 (19), MEK1 (17), MEK2 (18), and MEK5 (6). MKK7 displays 62, 49, 47, 43, 34, 34, and 35% amino acid identity to Hep, SEK1/MKK4, MKK6, MKK3, MEK1, MEK2, and MEK5, respectively. The amino acid sequence alignment of MKK7 with those related molecules is shown in Fig. 1B. These results show that MKK7 is a novel member of the MAPK kinase family of dual-specificity protein kinases.

Tissue Distribution of MKK7 mRNA—To determine the expression pattern of MKK7, we performed Northern blotting of multiple adult tissues. MKK7 is expressed at high levels in brain, lung, liver, skeletal muscle, kidney, and testis and at lower levels in the heart and spleen. Moreover, a second mRNA species of MKK7 can be detected in the testis (Fig. 2A). MKK7 mRNA is also expressed at high levels in wild type and sek1/mkk4 gene-deficient (25) embryonic stem cell (data not shown).

In situ hybridization of day 10.5, 12.5, 14.5, 16.5, and 18.5 embryos using a MKK7 antisense probe further confirmed that MKK7 is ubiquitously expressed at these stages of fetal development. On E10.5 (not shown) and E12.5 (Fig. 2, B and C), MKK7 was expressed at high levels in the brain, spinal cord, eyes, muscle, lungs, vertebrae, and intestine and at lower levels in the heart and liver. At later stages of embryogenesis (E14.5, E16.5, and E18.5) high levels of MKK7 expression were found in the brain, retina, and bone marrow. Interestingly, on E14.5, E16.5, and E18.5, MKK7 was highly expressed in skin, intestinal, and lung epithelium and the epithelial layers lining the olfactory cavity and developing teeth and whiskers (Fig. 2, D and E, and data not shown). It should be noted that Dro sophila hemipterous is required in epithelial cell movement and dorsal closure (29). The sequence homology between MKK7 and Drosophila hemipterous and high expression of MKK7 in epithelial tissues imply that, similarly to Hep, MKK7 might function in the development and/or morphogenesis of epithelial tissues.

MKK7 Activates SAPK/JNK in Transfected 293 Cells—MKK7 has high homology to Hep and MKK4/SEK1, both of which activate the SAPK/JNK pathway. To examine whether MKK7 can regulate the SAPK/JNK pathway, we co-transfected 293 cells with mammalian expression plasmids encoding MKK7 and HA epitope-tagged SAPKβ. Recombinant SAPKβ was immunoprecipitated from cell lysates and used in protein kinase assays with GST-c-Jun as substrate. Transfection of cells with MKK7 resulted in strong activation of SAPKβ (Fig. 3A, lane 2), whereas transfection with vector alone had no effect on SAPKβ activity (Fig. 3A, lane 1). The level of activa-
tion was comparable to that of cells transfected with MKK4/SEK1 (Fig. 3A, lane 4). A kinase-inactive form of MKK7 in which lysine 76 in the ATP binding domain was mutated to a glutamic acid (K76E) was unable to activate SAPKβ (Fig. 3A, lane 3), indicating that the kinase activity of MKK7 is required for the activation of SAPKs/JNKs in vivo. Western blot analysis
**FIG. 2. Tissue distribution of MKK7.**

A. Northern blotting for MKK7 mRNA expression in adult murine tissues. Standardized mouse multiple tissue Northern blots (2 μg/lane poly(A)^+ RNA) were probed with a full-length MKK7 probe. *Muscle*, skeletal muscle.

*B–E*, in situ hybridization. *kb*, kilobases. *B*, distribution of MKK7 message in E12.5 embryos detected by a full-length MKK7 antisense probe. *v*, vertebral column; *l*, lung; *b*, brain; *sc*, spinal cord; *i*, intestine; *h*, heart; *li*, liver. *C*, a MKK7 sense probe was used as a negative control for in situ hybridization.

*D*, high levels of MKK7 expression in whiskers (*w*), retina (*r*), skin (*s*), and brain (*b*) of E16.5 embryos. *E*, high levels of MKK7 expression in the skin (*s*), olfactory epithelium (*o*), and the developing tooth (*t*) of E16.5 embryos to demonstrate high MKK7 expression in epithelial sheets. Magnifications: *B–C*, × 10; *D* and *E*, × 30.
showed that SAPKβ was expressed at comparable levels (data not shown). Stimulation of 293 cells cotransfected with MKK7 and SAPKβ with IL-1β or LPS further increased SAPKβ activity by 1.5- and 2.2-fold, respectively, as compared with cells cotransfected with MKK7 and SAPKβ (data not shown).

To determine whether MKK7 phosphorylated SAPKs/JNKs at the physiological sites Thr-183 and Tyr-185, 293 cells were co-transfected with MKK7 and HA-tagged SAPKβ. Epitope-tagged SAPKβ was immunoprecipitated with an anti-HA mAb and blotted with a phospho-specific SAPK/JNK Ab and an Ab that is reactive against the SAPK/JNK protein. As shown in Fig. 3B, SAPKβ from cells co-transfected with wide type MKK7 but not from cells co-transfected with the kinase-inactive K76E mutant were specifically recognized by the phospho-specific SAPK/JNK Ab (upper panel, lane 2). Western blot analysis using an anti-HA Ab showed that epitope-tagged SAPKβ was expressed at similar levels in all cells (Fig. 3B, lower panel). This result indicates that MKK7 activates SAPK/JNK via phosphorylation at the Thr-183/Tyr-185 dual-phosphorylation motif.

The Kinase-inactive MKK7-K76E Mutant Inhibits IL-1β-, LPS-, and MEKK1-induced SAPK/JNK Activation—To determine whether endogenous MKK7 may play a role in SAPK/JNK activation in response to physiological stimuli, we tested whether the kinase-negative mutant of MKK7 (K76E) can block SAPK/JNK activation after IL-1β and LPS activation. 293 cells were co-transfected with the MKK7-K76E mutant and SAPKβ and then stimulated with either IL-1β or LPS. As shown in Fig. 4, the MKK7-K76E mutant and, as a control, the dominant negative MKK4-AL mutant (21) effectively blocked IL-1β-induced SAPK/JNK activation (Fig. 4A, lane 3). MKK4-AL mutant also blocked LPS-induced SAPKβ activation (data not shown). Western blot analysis using an anti-HA antibody revealed that SAPKβ was expressed at similar levels in all transfections (Fig. 4A, lower panel, and data not shown). Similarly, the kinase-inactive MKK7-K76E mutant inhibited LPS-stimulated SAPK/JNK activation (Fig. 4B, lane 3). Since either MKK4 or MKK7 kinase negative mutant completely inhibited IL-1β- or LPS-induced SAPKβ activation, it is possible that these mutants function by sequestering either upstream or downstream components in the SAPK/JNK pathway.

The MAP kinase kinase MEKK1 is a potent activator of MKK4/SEK1, which in turn phosphorylates and activates SAPKs/JNKs (11, 30). To determine whether MEKK1 is an upstream activator of MKK7 in the SAPK/JNK pathway, we analyzed the effect of the kinase-inactive MKK7-K76E mutant on MEKK1-induced SAPK/JNK activation. As shown in Fig. 4C, the MKK7-K76E mutant blocked MEKK1-induced SAPK/
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Fig. 5. ERK or p38 kinase activity in cells transfected with MKK7. A, 293 cells were transfected with vector (lane 1) or MKK7 (lane 2) and HA epitope-tagged ERK2 plasmid DNA. Alternatively, cells were transfected with ERK2 or ERK2 plus the MKK7-K76E mutant and stimulated with epidermal growth factor (30 ng/ml) for 10 min (lanes 3 and 4). Kinase assays were performed using PHAS-1 as substrate. B, 293 cells were co-transfected with either empty vector (lane 1) or MKK7 (lane 2) and HA epitope-tagged p38 kinase plasmids. Alternatively, cells were transfected with a p38 kinase plasmid and treated with 500 mM NaCl for 30 min (lane 3). P38 kinase assays were performed using an ATF-2 peptide as substrate.

JNK activation, suggesting that MKK7 functions downstream of MEKK1. These results demonstrate that MKK7 can relay signals from the physiological stimuli LPS and IL-1β to SAPK/JNK pathway and that MKK7 is a downstream target for MEKK1 activity in the SAPK/JNK signaling cascade.

**MKK7 Does Not Activate ERK2 or p38 Kinase in Transfected 293 Cells**—To examine whether MKK7 can also regulate the ERK and/or p38 kinase pathways, we co-transfected 293 cells with mammalian expression plasmids encoding murine MEKK7 and HA epitope-tagged ERK2 or HA-tagged p38 kinase. ERK2 or p38 kinase were immunoprecipitated from cell lysates and used in protein kinase assays with PHAS-1 or ATF-2 peptides as substrates. The addition of epidermal growth factor to 293 cells strongly activated ERK2 kinase activity (Fig. 5a, lane 1). However, no increase in ERK2 activity was observed when MKK7 was overexpressed in 293 cells (Fig. 5a, lanes 1 and 2). In addition, the MKK7-K76E mutant did not inhibit epidermal growth factor-induced ERK2 activation (Fig. 5a, lane 4). Similarly, whereas treatment of cells with NaCl strongly activated p38 kinase (Fig. 5b, lane 3), no increase in p38 kinase activity was observed when MKK7 was overexpressed in 293 cells (Fig. 5b, lanes 1 and 2). These data suggest that MKK7 does not play a role in the MAPK/ERK and p38 kinase pathways and that MKK7 is the second activator that relays stress signaling to the SAPK/JNK pathway.

Conclusions—Genetic studies in MKK4/SEK1 gene-deficient murine embryonic stem cells have demonstrated that a second activator of SAPKs/JNKs exists in vitro (25, 26) and that signaling pathways for SAPK activation are developmentally regulated during lymphopoesis (27). We have identified a new mitogen-activated kinase kinase MKK7 that specifically activates SAPKs/JNKs but not ERKs and p38 kinase. MKK7 is ubiquitously expressed in all adult and embryonic organs but displays high expression in epithelial tissues at later stages of fetal development. Moreover, our results show that MKK7 acts downstream of MEKK1 and that a kinase-inactive MKK7 mutant can inhibit SAPK/JNK activation in response to IL-1β and LPS. Further studies are needed to elucidate upstream signaling pathways and other physiological stimuli that activate MKK7.

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