M KK7 Is A Stress-activated Mitogen-activated Protein Kinase
Kinase Functionally Related to hemipterous

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Exposure of mammalian cells to stressful stimuli results in activation of the c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinases (SAPKs), a family of protein kinases related to mitogen-activated protein (MAP) kinase. JNK/SAPKs are activated by specific MAP kinase kinases (MKKs), one of which, MKK4/SEK1, has been characterized extensively. In Drosophila, the JNK/SAPK Basket (Bsk) and the MKK Hemipterous (Hep), are important for embryonic development. Loss of function of either gene inhibits dorsal closure, a morphogenetic movement in which the edges of the embryonic ectoderm move together over the amnioserosa. There is evidence that the Rho GTPases Rac and Cdc42 are also required for dorsal closure, suggesting that Rac or Cdc42 may regulate Hep and Bsk. We have identified MKK7, a murine homolog of Hep. MKK7 functionally rescues hep mutant flies. In fibroblasts, MKK7 is activated by stress and by the GTPase Rac1. MKK7 directly phosphorylates and activates JNK/SAPK. Thus, MKK7 is a homolog of hep and functions in a conserved signaling pathway involving JNK/SAPK and the GTPase Rac1.

Stressful stimuli, such as inflammatory cytokines, UV radiation, and protein synthesis inhibitors, and the Rho family GTPases Rac and Cdc42, activate two groups of MAP kinases in mammalian cells (1, 2). One group includes alternatively spliced isoforms of JNK/SAPKs and the other contains p38 MAP kinase and its relatives (1). JNK/SAPK and p38 are activated by dual specificity kinases known as MAP kinase kinase kinases (MKKs), by phosphorylation on a specific threonine and tyrosine residue within a TXY motif (1). Both JNK/SAPK and p38 can be activated by more than one MKK. JNK/SAPK is activated by MKK4/SEK1 and by additional unidentified activators (3, 4). Similarly, p38 is activated by both MKK3 and MKK4 (1). Roles for both JNK/SAPK and p38 in mediating growth arrest, apoptosis, or activation of immune responses have been proposed (1).

Recent evidence indicates that JNK/SAPKs may also mediate developmental processes. The Drosophila hemipterous (hep) and basket (bsk) genes encode an MKK and a JNK/SAPK relative, respectively (5–7). In vitro, Hep can phosphorylate and activate Bsk. Loss of function of either gene inhibits dorsal closure, a morphogenetic movement during early embryogenesis in which the edges of the ectoderm move together over the amnioserosa. This movement is accompanied by epithelial cell elongation and migration in the absence of cell proliferation, rearrangement, or death. There is evidence that Drosophila Rac and Cdc42 (DRaca, DCdc42) can induce gene expression dependent on hep, and expression of a dominant negative transgene of DRaca or DCdc42 (N17DRaca, N17Cdc42, respectively) during embryonic development inhibits dorsal closure (8–9). Thus the dorsal closure signaling pathway includes Hep, Bsk, and a Rho family GTPase.

Here we report the identification of MKK7 and show it is a murine homolog of Hep that functionally rescues hep mutant flies. In cultured mammalian fibroblasts, MKK7 is a physiological regulator of JNK/SAPK. Fractions of osmotically shocked NIH3T3 cell lysates, which contain the major peak of JNK/SAPK activating activity, also contain MKK7, while MKK4/SEK1 coincides with a smaller peak of activity. Moreover, MKK7 directly phosphorylates and activates JNK/SAPK, and MKK7 activation is mediated by the GTPase Rac1. Thus, MKK7 is a critical component of the JNK/SAPK stress response pathway.

EXPERIMENTAL PROCEDURES

Screening and Cloning—The COOH-terminal fragment of MKK7 was identified in a yeast two-hybrid library screen using full length wild-type human MKK1a (10) as bait, and a mouse day 9.5–10.5 embryo cDNA library (11). Fourteen clones from 1.2 × 108 transformants were sequenced. Clone MKKIP85a represented a putative novel MKK COOH-terminal fragment of 128 amino acids, which was used to probe a mouse day 16 embryo cDNA library (Novagen). This screen yielded three clones, which contained only 71 additional amino acids of 5′-coding sequence. A strategy of 5′-rapid amplification of cDNA ends PCR using mouse brain RNA subsequently yielded 141 additional amino acids of 5′-coding sequence. A mouse brain cDNA library (Stratagene) ultimately yielded two clones with diverging 5′-sequence, an initiator methionine and upstream in-frame stop codons (MKK7a and MKK7b).
Full-length MKK7α was generated by overlap PCR (12). Confirmation that the full MKK7 sequence was present in tissues was determined by standard reverse transcriptase PCR using mouse brain and kidney RNA templates. Sequence of forward and reverse strands was confirmed. MKK7α was used for all studies presented in this report.

Transgene Constructs—UBhek has been described (5). To construct UBMKK7, UBMKK4/SEK1, and UBXMEK2, a NotI fragment containing a ubiquitin promoter-x-hep 70 3′-untranslated region, where X represents either MKK7, MKK4/SEK1, or XMEK2 cDNAs, was cloned into the pCaSpeR4 transformation vector (13). The size and origin of the restriction fragments containing the coding region for MKK7, MKK4/SEK1, and XMEK2 are as follows: 1.3-kb MKK7a EcoRI fragment from pCR1.28-kb XhoI fragment from a pXMK-SK1 vector, 1.8-kb EcoRI fragment from a pXM-XMEK2 vector. P element-mediated germ line transformation followed standard protocols (15).

Genetics—Genetic markers and balancer chromosomes have been described (16). Novel hep alleles were obtained by imprecise excision of a P element from the hep′ stock (5). The ability of either MKK7, MKK4/SEK1, or XMEK2 to rescue hep zygotic lethality was tested as follows: hep′/MF6, rye/po/po females were mated to w/Y; pUB-X/TM3 or w/Y; pUB-X/p(UB-X) males. X represents either hep, MKK7, MKK4/SEK1, or XMEK2 cDNAs. Rescue activity was calculated as the percent of hep/Y; UB-X+/+ (rescued) compared with Pfinish/Y; UB-X+ (control) males. For each cross, two independent lines were tested and showed similar results. At least 50 control males were counted in each experiment.

Immunoprecipitations—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Plasmid DNA was transfected with LipofectAMINE (Life Technologies, Inc.), and cells were harvested 48 h after transfection. Total amount of plasmid DNA was kept constant and adjusted with pCs3 vector DNA. For immuno-precipitations, cells were washed with phosphate-buffered saline and lysed on ice in a buffer containing 1% Triton X-100, 10 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM NaF, 0.2 mM Na3VO4, 0.1% protease inhibitor, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. MT-JNK1 and MT-MKK7 were immunoprecipitated for 1 h at 4 °C with anti-Myc (9E10). Immune complexes were recovered using Pansorbin coated with goat-anti-mouse IgG. HA-SEK1 was immunoprecipitated with anti-HA (12CA5) and recovered with protein A-Sepharose beads (Sigma). Complexes were washed three times with lysis buffer and once with 10 mM Pipes (pH 7.0), 0.1 M NaCl, and 1% aprotinin and resuspended in 10 μl of kinase reaction buffer containing 25 mM Pipes (pH 7.4), 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, 0.1 mM Na3VO4, 2.5 μM ATP, 100 μM unlabelled ATP, and 2 μg of the indicated substrate. Assay of protein kinase activities was as described (3).

Following SDS-PAGE, autoradiography and autoradiography, proteins were quantitated with a PhosphorImager. MKK7 was detected with antisera raised against a GST fusion of the COOH-terminal 100 residues (antisera 3936). All experiments shown were repeated two to five times with similar results.

Phosphorylation—SacI-stimulated (0.4 M, 30 min) or unstimulated NIH3T3 cells (10 × 10^6) were harvested as described (17) except that the lysis buffer contained 25 mM Tris (pH 7.3), 10 mM β-glycerophosphate, 1.5 mM EDTA, 1.5 mM EGTA, 1 mM Na3VO4, 1 mM benzamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM dithiothreitol, and 200 μM microcystin. 100,000 × g lysates from cells were applied to a Mono S column after passing over a Mono Q column. Chromatography conditions were as described (18), except that the salt gradient was 30 ml. Fractions were assayed for kinase activity and analyzed by SDS-PAGE and autoradiography. Phosphorylated GST and MT-MKK7 were immunoprecipitated with anti-Myc (9E10). Immunoassays were performed as described (18), except that the salt gradient was 30 ml. Fractions were assayed for kinase activity and analyzed by SDS-PAGE and autoradiography. Phosphorylated GST and MT-MKK7 were immunoprecipitated with anti-Myc (9E10). Immunoassays were performed as described (18), except that the salt gradient was 30 ml. Fractions were assayed for kinase activity and analyzed by SDS-PAGE and autoradiography. Phosphorylated GST and MT-MKK7 were immunoprecipitated with anti-Myc (9E10).

RESULTS AND DISCUSSION

Two alternatively spliced MKK7 cDNAs were cloned from various libraries. The two cDNAs differ at their 5′-ends and encode two proteins with a common COOH-terminal 377-residue region containing all the hallmarks of dual specificity kinases (Fig. 1A). In addition, JNK is a major transcript of approximately 4 kb was present in all tissues analyzed and was more abundant in skeletal muscle, heart, brain, and testis than in spleen, kidney, lung, or liver (Fig. 1A). Additional weaker transcripts of 7 and 9 kb were also noted in all tissues. These may represent alternative processing of transcripts from a single gene or other closely related protein kinases. The abundant short 2-kb transcript found in testis may represent a germ cell-specific transcript.

MKK7 is closely related to Hep, sharing 57% identity overall and 71% identity within the kinase domain (Fig. 1C). For comparison, MKK4/SEK1 is 48% identical with Hep over the
full sequence. To test whether MKK7 could function in place of Hep in Drosophila development, we expressed hep and MKK7 as transgenes under the control of the ubiquitin promoter. The transgenes were introduced into flies carrying nine different lethal hep alleles. Expression of hep rescued all alleles, allowing complete development to viable, fertile, adult males (Table I). Rescue was specific, since flies mutant for Dsor1, another MKK (20), were not rescued. Expression of MKK7 rescued 42 and 62%, respectively, of animals carrying two lethal hep alleles (rh1 and rh99, respectively). Some fully fertile and viable males were obtained, indicating complete rescue. However, the majority of adult males rescued by MKK7 had defects in the dorsal thorax and in the development and rotation of the anal plate and genital arches (data not shown). This spectrum of defects is reminiscent of those displayed by a hypomorphic, viable hep allele (5). Moreover, seven hep alleles were not rescued significantly. This suggests that MKK7 can substitute for hep at some stages of development but not others. MKK7 was quantitatively more efficient than mammalian or Xenopus MKK4/SEK1 in rescuing the viability of hep alleles (Table I), confirming the relatedness of MKK7 and hep.

We examined the binding properties of MKK7. We used a yeast two-hybrid assay to examine the ability of MKK7 to associate with JNK1 (SAPK\(\alpha\)) p38, or ERK2 MAP kinases. MKK7 interacted only with JNK1.\(^2\) In a separate assay, bacterially expressed GST or GST-MKK7 coupled to glutathione-Sepharose beads was incubated with \(\text{\[^{35S}\]methionine-labeled, in vitro translated JNK1, p38, or ERK2. Following incubation, samples were washed and analyzed by SDS-PAGE and autoradiography. GST-MKK7 binds strongly to JNK1 and weakly to ERK2 (Fig. 2). This demonstrates that MKK7 can associate with JNK1 in vitro.

To investigate the substrate specificity and activation of MKK7, we expressed epitope-tagged MKK7 in NIH3T3 cells and measured its activity in vitro. Cells were transiently transfected with either Myc-tagged MKK7, HA-tagged MKK4/SEK1, or vector. Cells were left untreated or stimulated with PDGF, anisomycin, or NaCl. Immunoprecipitated MKK7 and MKK4/SEK1 were assayed for their ability to phosphorylate the substrate proteins His-SAPK\(\alpha\) (JNK2), GST-p38, or a catalytically inactive mutant of ERK2, His-ERK2 K52R (Fig. 3). Both MKK7 and MKK4/SEK1 were expressed and immunoprecipitated, as judged by Western blotting (Fig. 3B). MKK7 and MKK4/SEK1 were able to phosphorylate SAPK\(\alpha\) better than p38 or ERK2 K52R (Fig. 3B). The inability of MKK7 to phosphorylate ERK2 K52R in this assay suggests that the weak association noted between GST-MKK7 and ERK2 in Fig. 2 may be nonspecific.

To determine whether MKK7 phosphorylates SAPK\(\alpha\) and p38 at the physiological sites, we measured SAPK\(\alpha\) and p38 activities. Both MKK7 and MKK4/SEK1 were able to efficiently activate SAPK\(\alpha\), using GST c-Jun as a substrate, and weakly activate p38, using GST ATF2 as a substrate (Fig. 3C). Consistent with their abilities to phosphorylate stress-activated kinases, MKK7 and MKK4/SEK1 were activated in stressed cells. Both kinases were activated by osmotic stress or partial inhibition of protein synthesis (by anisomycin) and were not activated by a mitogen, PDGF (Fig. 3D). MKK7 was consistently activated more by osmotic stress than by anisomycin, whereas MKK4/SEK1 was activated equally (Fig. 3D). This may indicate differences in upstream activators. We have also observed activation of MKK7 in response to UV.\(^2\) These results show that MKK7 is activated by extracellular stresses and can bind to and activate SAPK\(\alpha\) in vitro.

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\(^2\) P. M. Holland, unpublished results.
JNK1 activity was measured in immune complexes, using GST with MKK7 and treated with anisomycin or left unstimulated. Cells were transfected with pCS3±MT-JNK1 (SAPK) and either wild-type pCS3±MT-MKK7, MKK7 S3A (S271A, T275A, S277A), or empty vector. Cells were treated with anisomycin (10 μg/ml; 20 min) or left untreated. Immunoprecipitated JNK1 activity was measured using GST c-Jun as a substrate. JNK1 expression was analyzed by immunoblotting with anti-JNK1 (Santa Cruz). MKK7 and pcDNA3-MKK4 (Promega TNT kit) were immunoblotted with anti-MKK7 and anti-SEK1. The specificity of the antibodies was tested using translated pCS3±MT-JNK1 in vitro and either wild-type MKK7 and MKK4/SEK1 (21–23), inactive mutants of MKK7 (3936), respectively. GST c-Jun phosphorylation is expressed as fold increase with respect to JNK1 transfected cells without stimulus. The experiment was repeated two times with similar results.

![A New JNK/SAPK Activator, MKK7, Is a hemipterous Homolog](image)

We investigated whether MKK7 could activate JNK/SAPK in cells by performing transient co-transfection assays in NIH3T3 cells. Cells were transfected with epitope-tagged JNK1 alone or with MKK7 and treated with anisomycin or left untreated. JNK1 activity was measured in immune complexes, using GST c-Jun as a substrate. Wild-type MKK7 potentiated JNK1 activity even in the absence of stimulus (Fig. 4A). An S3A mutant (S271A, T275A, S277A), which lacks kinase activity in vitro, did not increase JNK1 activity in cells. A similar result was observed with a K149M mutant of MKK7. Unlike inactive mutants of MKK4/SEK1 (21–23), inactive mutants of MKK7 are not dominant inhibitors of JNK1 activation (Fig. 4A). It is possible that MKK7 activity in cells is restricted by negative regulators that can be overcome by overexpression.

To address whether endogenous MKK7 is regulated by stresses, osmotically shocked untransfected NIH3T3 cells were co-transfected with wild-type pCS3±MT-MKK7 and either pcGT-RacV12, RacV12N17, or empty vector. Cells were treated with NaCl (0.4 M; 30 min), and MKK7 was immunoprecipitated and assayed for activity as in Fig. 3. Expression of Rac and immunoprecipitated MKK7 was determined by immunoblotting with anti-T7 (Novagen) and anti-MKK7 (3936), respectively. GST c-Jun phosphorylation is expressed as fold increase with respect to MKK7 transfected cells without stimulus. The experiment was repeated two times with similar results. We investigated whether MKK7 could activate JNK/SAPK in cells by performing transient co-transfection assays in NIH3T3 cells. Cells were transfected with epitope-tagged JNK1 alone or with MKK7 and treated with anisomycin or left untreated. JNK1 activity was measured in immune complexes, using GST c-Jun as a substrate. JNK1 expression was analyzed by immunoblotting with anti-JNK1 (Santa Cruz). MKK7 and pcDNA3-MKK4 (Promega TNT kit) were immunoblotted with anti-MKK7 and anti-SEK1. The specificity of the antibodies was tested using translated pCS3±MT-JNK1 in vitro and either wild-type MKK7 and MKK4/SEK1 (21–23), inactive mutants of MKK7 (3936), respectively. GST c-Jun phosphorylation is expressed as fold increase with respect to JNK1 transfected cells without stimulus. The experiment was repeated two times with similar results.

![Effect of Rac1 mutants on MKK7 activity. NIH3T3 cells were co-transfected with wild-type pCS3±MT-MKK7 and either pcGT-RacV12, RacV12N17, or empty vector. Cells were treated with NaCl (0.4 M; 30 min), and MKK7 was immunoprecipitated and assayed for activity as in Fig. 3. Expression of Rac and immunoprecipitated MKK7 was determined by immunoblotting with anti-T7 (Novagen) and anti-MKK7 (3936), respectively. GST c-Jun phosphorylation is expressed as fold increase with respect to MKK7 transfected cells without stimulus. The experiment was repeated two times with similar results.](image)
of JNK/SAPK in MKK4/SEK1\(^{-/-}\) cells still occurs in response to osmotic shock and UV irradiation, but not in response to anisomycin or heat shock (24, 25). Work from M. Kracht also shows that MKK7 and not MKK4/SEK1 is the major JNK/SAPK activator in IL-1 treated rabbits. These data imply that activation of JNK/SAPK by different environmental stimuli occurs selectively through different MKKs. The greater ability of MKK7 to complement hom over MKK4 also implies hep in the Rac-dependent cytoskeletal rearrangements of dorsal closure, and MKK7 is activated by Rac in fibroblasts, yet recent studies have suggested, or whether Rac regulates the cytoskeleton directly and Hep is needed for a prior signaling step. The properties of MKK7 suggest that a single MKK can be important both for normal development and for stress responses. MKK7 can partially substitute for hep in the Rac-dependent cytoskeletal rearrangements of dorsal closure, and MKK7 is activated by Rac in fibroblasts, yet recent studies have suggested that Rac-dependent cytoskeletal changes in fibroblasts are independent of MKK7 and JNK/SAPK (30–31). It will be important to test whether the cytoskeletal changes in Drosophila dorsal closure are relayed by Hep from Rac, as has been suggested, or whether Rac regulates the cytoskeleton and whether Rac regulates the cytoskeleton directly and Hep is needed for a prior signaling step.

Note Added in Proof—The molecular cloning of a human homolog and alternatively spliced forms of murine MKK7 was recently reported by Tournier et al. (32).

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