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The M KK7 Gene Encodes a Group of c-Jun NH2-Terminal Kinase Kinases

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The c-Jun NH2-terminal protein kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) group and is an essential component of a signaling cascade that is activated by exposure of cells to environmental stress. JNK activation is regulated by phosphorylation on both Thr and Tyr residues by a dual-specificity MAPK kinase (MAPKK). Two MAPKKs, MKK4 and MKK7, have been identified as JNK activators. Genetic studies demonstrate that MKK4 and MKK7 serve nonredundant functions as activators of JNK in vivo. We report here the molecular cloning of the gene that encodes MKK7 and demonstrate that six isoforms are created by alternative splicing to generate a group of protein kinases with three different NH2 termini (α, β, and γ isoforms) and two different COOH termini (1 and 2 isoforms). The MKK7α isoforms lack an NH2-terminal extension that is present in the other MKK7 isoforms. This NH2-terminal extension binds directly to the MKK7 substrate JNK. Comparison of the activities of the MKK7 isoforms demonstrates that the MKK7α isoforms exhibit lower activity, but a higher level of inducible fold activation, than the corresponding MKK7β and MKK7γ isoforms. Immunofluorescence analysis demonstrates that these MKK7 isoforms are detected in both cytoplasmic and nuclear compartments of cultured cells. The presence of MKK7 in the nucleus was not, however, required for JNK activation in vivo. These data establish that the MKK4 and MKK7 genes encode a group of protein kinases with different biochemical properties that mediate activation of JNK in response to extracellular stimuli.

Mitogen-activated protein kinases (MAPKs) are components of pathways that relay signals to particular cell compartments in response to a diverse array of extracellular stimuli (38, 42, 63, 83). Activated MAPK can translocate to the nucleus and phosphorylate substrates, including transcription factors, thereby eliciting a biological response. At least three groups of MAPKs have been identified in mammals: ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase; also known as stress-activated protein kinase), and p38 MAPK (also known as cytokine-suppressive anti-inflammatory drug-binding protein). ERK contributes to the response of cells to signals initiated by many growth factors and hormones through a Ras-dependent pathway (63). In contrast, JNK and p38 MAPK are activated by environmental stresses, such as UV radiation, osmotic shock, heat shock, protein synthesis inhibitors, and lipopolysaccharide (38, 83). The JNK and p38 MAP kinases are also activated by treatment of cells with proinflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (38, 83). MAPKs are involved in the control of a wide spectrum of cellular processes including growth, differentiation, survival, and death (38, 63).

MAPKs are activated by conserved protein kinase signaling modules which include a MAPK kinase kinase (MAP3K) and a dual-specificity MAPK kinase (MAPKK). The MAP3K phosphorylates and activates the MAPKK which, in turn, activates the MAPK by dual phosphorylation on threonine and tyrosine residues within a Thr-Xaa-Tyr motif located in protein kinase subdomain VIII (38, 63). Separate protein kinase signaling modules are used to activate different groups of MAPKs.
nase (LZK) (64). Binding sites for the Rho family GT-Pases Cdc42 and Rac1 have been described for MLK1, MLK2, and MLK3 (but not DLK or LZK) (7). It has also been reported that MEKK1 and MEKK4 (but not MEKK2 or MEKK3) bind directly to the Rho family GT-Pases Cdc42 and Rac1 (20). These interactions between Rho GT-Pases and MAPKKks may contribute to the effects of Rho GT-Pases on JNK activation. MAPKK protein kinases that do not interact with Rho GT-Pases may mediate the effects of Rho-independent signals that lead to JNK activation.

Several MAPKKs have been identified. ERK is activated by MEK1 and MEK2 (1); p38 MAPK is activated by MEK3 (13), MKK4 (13, 45), and MKK6 (28, 53, 61, 68); JNK is activated by MKK4 (13, 45, 65). The existence of JNK activators distinct from MKK4 was suggested by chromatographic fractionation of cell extracts (48, 52) and by the results of targeted disruption of the MKK4 gene (58, 87). We (76) and others (21, 34, 36, 44, 46, 55, 77, 84, 89) have recently characterized the novel JNK activator MKK7. In contrast to MKK4, which activates both JNK and p38 MAPK, the MKK7 protein kinase selectively activates only JNK. Interestingly, the two JNK activators D-MKK4 (Drosophila MKK4) (29) and Hep/D-MKK7 (26, 67) are conserved in Drosophila. Genetic analysis demonstrates that D-MKK4 and Hep/D-MKK7 serve nonredundant functions in the fly (38). Similarly, gene disruption experiments demonstrate that MKK7 is an essential gene in the mouse, indicating that MKK7 is unable to fully substitute for the function of MKK4 (24, 57, 60, 87). These data demonstrate that MKK4 and MKK7 serve nonredundant functions as activators of the JNK MAP kinases in vivo.

We report here the molecular cloning of the MKK7 gene and the characterization of MKK7 isoforms that are created by alternative splicing. The MKK7 isoforms are differentially activated by upstream signals, and their regulation differs from that of the MKK4 isoforms. These data establish that JNK activation is mediated by a family of protein kinases that are formed by the alternative splicing of two genes.

**MATERIALS AND METHODS**

**Materials.** Human TNF-α and IL-1α were from Genzyme Corp. [γ-32P]ATP was obtained from Amersham Corp. Recombinant glutathione S-transferase (GST)–c-Jun and GST-JNK1 were purified from bacteria as described elsewhere (12). Mammalian expression vectors for MKK4 isoforms (13) and MKK7 isoforms were subcloned into the mammalian expression vectors pCDNA3 (Invitrogen) and pEBG (86). The Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Immunex Corp.) was fused to the NH2 terminus of the MKK7 isoforms by inserting overlapping PCR (35). A nuclear export signal (NES) sequence (23) encoding an additional 34 amino acids was inserted by N. Abe (67). The expression vectors pEBG-KHS (78), pSRE-HPK1 (37), pMT2-MEKK3 (15), pCDNA1-MTK1/MEEK4 (71), pCDNA3-MK3-LK3 (74), and pSRE-DL (33) were provided by J. Blenis, T. Tan, U. Siebenlist, H. Saito, S. Gutfnick, and J. Avruch, respectively.

**Molecular cloning of MKK7 isoforms.** Genomic DNA clones corresponding to the MKK7 locus were isolated by screening a λ FIXI murine genomic library (Stratagene, Inc.), using the MKK7α cDNA as a probe. The genomic sequence of MKK7 was obtained with an Applied Biosystems model 373A machine. To determine the 5′ and 3′ untranslated regions, cDNA clones have been deposited in GenBank with accession no. U93030, AF006943, AF006944, AF006948, and AF006947.

**RESULTS**

**Molecular cloning of MKK7 isoforms.** MKK7 has recently been identified as a specific activator of the JNK group of MAPKs. Northern blot analysis demonstrated the presence of at least two MKK7 mRNAs (2.2 and 4.2 kb) in adult murine tissues (76), an observation that suggested MKK7 may be expressed as a group of alternatively spliced isoforms. To characterize members of the MKK7 group, we exhaustively screened a mouse testis cDNA library by using our original biotinylated and used as the hybridization probe (30). The FISH and 4,6-diamidino-2-phenylindole (DAPI) staining were recorded on film. The assignment of the FISH mapping data with chromosomal bands was achieved by superimposing the FISH signals with images of the DAPI-banded chromosomes. Tissue culture and transfection assays. COS-7 and 293 cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, 100 U of penicillin and streptomycin per ml, and 100 U of leupeptin per ml in a humidified environment of 5% CO2. Transient transfections were performed with the LipofectAMINE reagent (Life Technologies) according to the manufacturer’s recommendations. After 36 h, the cells were serum starved for 1 h and treated with UV-C (80 J/m2), anisomycin (5 μg/ml); TNF-α (20 ng/ml), or IL-1α (2 to 10 ng/ml) for 30 min prior to lysis. Cell extracts were prepared in lysis buffer (20 mM Tris [pH 7.4], 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 × g (15 min at 4°C). The concentration of total soluble protein in the supernatant was quantitated by the Bradford method (Bio-Rad).

**MAPK kinase assays.** GST-tagged MKK7 proteins were isolated by incubation with glutathione (GSH)-Sepharose (Pharmacia-LKB Biotechnology) in lysis buffer (4 h at 4°C). The beads were washed five times with lysis buffer, and the presence of bound JNK was examined by immunoblot analysis.

**Protein kinase assays.** Epitope-tagged MKK7 proteins were immunoprecipitated from cell extracts by incubation for 3 h at 4°C with the Flag-specific monoclonal antibody M2 (IBI-Kodak) bound to protein G-Sepharose beads (Pharmacia-LKB Biotechnology). GST-tagged MAPKK was isolated by incubation for 3 h at 4°C with GSH-Sepharose beads (Pharmacia-LKB Biotechnology). The complexes were washed twice with lysis buffer and three times with kinase buffer (25 mM HEPES [pH 7.4], 25 mM β-glycerophosphate, 25 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate). MAPKK activity was measured at 30°C for 15 min in 30 μl of kinase buffer containing 0.5 μg of GST–c-Jun, and 50 μM [γ-32P]ATP (10 Ci/mmol; 1 Ci = 37.6 GBq). The reactions were terminated by addition of Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, and identified by autoradiography. The incorporation of [32P]phosphate into GST-c-Jun was quantitated by PhosphorImager analysis.

JNK protein kinase assays were performed with extracts of cells transfected with HA-JNK1. The JNK was immunoprecipitated with an antibody to the HA epitope tag (12CA5; Boehringer Mannheim). Protein kinase activity was measured in the immune complex with c-Jun as the substrate (12).

**Western blot analysis.** Proteins were resolved by SDS-PAGE (10% gel), electrophoretically transferred to an Immobilon-P membrane (Millipore Inc.), and monoclonal antibodies (1:500) to Flag (M2) and phospho-c-Jun (1:1000; Oncogene Sciences). Immune complexes were detected by enhanced chemiluminescence (Lumiglo; Kirkegaard & Perry Laboratories).

**Immunofluorescence microscopy.** COS-7 cells were seeded onto glass coverslips coated with poly-l-lysine (Sigma Chemical Co.; 36 h after transfection, the cells were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline, rinsed with water, and then permeabilized with 0.2% Triton X-100 in PBS. After incubation for 15 min with 3% (wt/vol) bovine serum albumin (BSA) in PBS, the coverslips were incubated for 1 h with primary antibodies in PBS with 3% BSA. The primary antibodies were rabbit anti-MKK4 (K-18; 1:100; Santa Cruz Biotechnology), goat anti-MKK7 (E-18; 1:100; Santa Cruz Biotechnology), rabbit anti-phosphorylated (on Thr-183 and Tyr-185) JNK (phospho-JNK) (9251; 1:100; Cell Signaling Technology), and monoclonal antibodies (1:500 to Flag M2) and HA (12CA5). Immune complexes were detected with Texas red-conjugated goat anti-mouse immunoglobulin (Ig) (Jackson Immunoresearch Laboratories) and fluorescein-conjugated anti-rabbit Ig. Fluorescein-conjugated anti-rabbit Ig antibodies (1:100; Jackson Immunoresearch, Inc.) in 3% BSA in PBS. After nuclei were stained for 1 min with DAPI (1:10,000; Molecular Probes, Inc.), the coverslips were mounted in Vectashield (Vector Laboratories, Inc.). All procedures were performed at room temperature. Fluorescence microscopy was performed with a Zeiss Axioskop microscope.

**Nucleotide sequence accession numbers.** The sequences of the murine MKK7 cDNA clones have been deposited in GenBank with accession no. U93030, AF006943, AF006944, AF006945, AF006946, and AF006947.

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MKK7 clone (MKK7α1) as a probe. This analysis led to the identification of additional isoforms: MKK7α2, MKK7β1, MKK7β2, MKK7γ1, and MKK7γ2 (Fig. 1). The predicted amino acid sequences are identical except at the NH2 terminus (α, β, and γ isoforms) and at the COOH terminus (1 and 2 isoforms).

We isolated a murine genomic clone that encodes the MKK7 protein kinase isoforms. FISH analysis led to the mapping of the gene to mouse chromosome 11 region A2 (Fig. 2). Sequence analysis of the gene demonstrated the presence of 14 exons (Fig. 3). Sequence comparison of the gene with cDNA encoding the MKK7 isoforms demonstrated that these isoforms are created by the usage of alternative exons. This analysis indicated that MKK7 is a complex gene that expresses a group of MKK7 protein kinases.

Initiation codons in frame with upstream termination codons are located in separate exons in the 5' region of the MKK7 gene. The α isoforms are created by using an initiation codon located in exon 4 in frame with an upstream termination codon located in exon 3 (Fig. 3). In contrast, the initiation codon (together with an upstream in-frame termination codon) used to create the β and γ isoforms is located in exon 1. Alternative splicing which includes or excludes exon 2 distinguishes the β and γ isoforms (Fig. 3).

Alternative splicing in the 3' region of the gene changes the usage of termination codons to create a truncated COOH terminus (1 isoforms) and an extended COOH terminus that includes 33 additional amino acids (2 isoforms). The truncated COOH terminus results from the presence of an in-frame termination codon and a polyadenylation signal in exon 13 (Fig. 3). Alternative splicing within exon 13 immediately prior to this termination codon fuses the open reading frame to sequences located in exon 14. This fusion creates the longer COOH terminus that is characteristic of the 2 isoforms. A termination codon and a polyadenylation signal are located in exon 14.

**Comparison of protein kinase activities of MKK7 isoforms.** We used a coupled protein kinase assay in vitro to examine the
protein kinase activities of MKK7 isoforms that were isolated from transfected mammalian cells. This assay employs bacterially expressed JNK1 as a substrate for the MKK7 protein kinases. The activation of JNK1 by MKK7 was examined by using bacterially expressed c-Jun as a substrate for JNK. Control experiments indicated that MKK7 does not phosphorylate c-Jun (data not shown). These assays demonstrated that the activities of the MKK7 β and MKK7 γ isoforms were similar and that the alternative splicing at the COOH terminus of MKK7 (1 and 2 isoforms) did not markedly alter protein kinase activity (Fig. 4). In contrast, activities of the MKK7α isoforms were lower than those of the MKK7β and MKK7γ isoforms. Quantitation of the MKK7 activity by PhosphorImager analysis indicated that MKK7β1 was 37-fold more active than MKK7α1.

The difference in protein kinase activity of the MKK7 isoforms was detected under basal conditions (Fig. 4). To determine whether similar differences could be detected following activation, we examined the effect of a strong activator of the JNK signaling pathway (MEKK1). We found that MEKK1 caused marked activation of all the MKK7 isoforms. The largest increase was observed for MKK7α1 and MKK7α2, which were activated by 34- and 20-fold, respectively (Fig. 4). The MKK7β and MKK7γ protein kinases were activated more modestly (three- fivefold). These data demonstrate that the MKK7β and MKK7γ isoforms are more active than MKK7α isoforms (under basal conditions and following activation) but that the MKK7α isoforms are more inducible following stimulation. The low basal activity of the MKK7α isoforms accounts, in part, for their greater activation.

Together, these data demonstrate that the activity of MKK7 isoforms is affected by alternative splicing of the NH₂-terminal region but not by alternative splicing of the COOH-terminal region. However, the inclusion or exclusion of exon 2 within the NH₂-terminal region of MKK7 (β and γ isoforms) did not alter MKK7 protein kinase activity in these assays.

The NH₂-terminal region of MKK7β interacts with JNK. The observation that activities of the MKK7α isoforms were lower than those of the MKK7β and MKK7γ isoforms suggests that these MKK7 isoforms may differentially interact with the substrate JNK. To test this hypothesis, we examined the interaction of MKK7α and MKK7β isoforms with JNK following

FIG. 3. Schematic representation of the structure of the MKK7 gene. The MKK7 gene is formed by 14 exons. The alternative splicing that creates the α, β, γ, 1, and 2 isoforms of MKK7 is illustrated. The coding (black) and noncoding (grey) regions and excluded exons (striped) are shown. Initiation codons (ATG), termination codons (+), and polyadenylation signals (●) are indicated.
expression in COS-7 cells. We precipitated the MKK7 protein kinases from cell lysates, and examined the presence of JNK1 in the precipitates by immunoblot analysis (Fig. 5A). These assays demonstrated that MKK7β isoforms, but not MKK7α isoforms, coprecipitated with JNK. This binding interaction may contribute to the higher activities of MKK7β isoforms than of MKK7α isoforms (Fig. 4).

The MKK7β and MKK7α isoforms differ structurally by virtue of an NH₂-terminal extension that is present in MKK7β but not MKK7α. This NH₂-terminal region of MKK7β therefore accounts for the binding interaction observed between JNK and MKK7β. The mechanism by which this NH₂-terminal region alters the binding to JNK is unclear. The NH₂-terminal region may act indirectly by altering the conformation of another region of the MKK7 protein kinase that binds JNK. Alternatively, JNK may bind directly to the NH₂ terminus of MKK7β. To test the latter hypothesis, we expressed the NH₂-terminal region of MKK7β (residues 1 to 73) as a GST fusion protein in bacteria. This recombinant protein was found to bind JNK (Fig. 5B). These data demonstrate that the MKK7β isoforms bind JNK and indicate that this is mediated, in part, by a direct interaction between JNK and the NH₂-terminal region of MKK7β that is absent in MKK7α. The presence of a region in the NH₂ terminus of MKK7β that binds JNK is consistent with previous studies that have implicated the NH₂-terminal region of MAPKK in the determination of signaling specificity to distinct MAPK isoforms (4).

Regulation of MKK7 isoforms by MAPKKKs. The protein kinases MEKK1, MLK3, and DLK preferentially activate JNK (32, 33, 50, 62, 74, 75, 86). MEKK3 activates both JNK and ERK (6, 11, 15), while MEKK4 has been reported to activate both JNK and p38 MAPK (25, 71). Since MEKK1 causes differential activation of MKK7 isoforms (Fig. 4), we examined whether other MAPKKKs, including members of the MEKK and MLK groups (19), could also cause differential activation of MKK7 isoforms.

To test the effects of various MAPKKK isoforms on activation of MKK7 isoforms, we examined MEKK and MLK protein kinases in cotransfection assays. Control experiments demonstrated that MEKK1, MEKK3, MLK3, and DLK caused similar increases in JNK activity (Fig. 6). However, MEKK4 caused no JNK activation (Fig. 6), in contrast to a previous report (25). Instead, MEKK4 selectively activated p38 MAPK (data not shown). These data identify MEKK4 as an activator of the p38 MAPK pathway (71).

To compare the levels of activation of MKK4 and MKK7 isoforms by different MAPKKK isoforms, we performed coupled protein kinase assays. MKK4 and MKK7 activity was detected with bacterially expressed JNK1 as the substrate, and JNK activation was assessed by measurement of the phosphorylation of c-Jun. Protein immunoblot analysis demonstrated that similar amounts of MKK7 were examined in all assays (Fig. 7). While MEKK1, MEKK3, MLK3, and DLK efficiently activated JNK (Fig. 6), these MAPKKKs displayed differences in the ability to activate MKK4 and MKK7 isoforms. For example, MEKK1 was the most potent activator of MKK7 isoforms, whereas MEKK3 was the most potent activator of MKK4 isoforms (Fig. 7). MEKK1, MLK3, and DLK efficiently activated JNK (Fig. 6), in contrast to a previous report (25). These data identify MEKK4 as an activator of the p38 MAPK pathway (71).
vate JNK (Fig. 6), we found that MEKK4 did not activate any of the MKK4 or MKK7 isoforms (Fig. 7).

Regulation of MKK4 and MKK7 isoforms by STE20-like protein kinases. Several STE20-like protein kinases have been reported to be capable of activating the JNK pathway (19). Among them, KHS (78) and HPK1 (37, 41, 79) appear to be specific for the JNK signaling pathway. We therefore examined the abilities of KHS and HPK1 to activate MKK4 and MKK7 isoforms. Cells were transiently transfected with expression vectors encoding epitope-tagged MKK4 or MKK7 isoforms, together with expression vectors for either KHS or HPK1. Control experiments demonstrated that the KHS and HPK1 protein kinases caused similar levels of JNK activation (Fig. 6). The amount of MKK4 and MKK7 protein expression was examined by Western blot analysis (Fig. 8). The MKK4 and MKK7 protein kinases were immunoprecipitated, and their activities were measured by a coupled protein kinase assay.

Regulation of MKK4 and MKK7 protein kinases by extracellular stimuli. To identify the nature of the MKKs involved in the regulation of the JNK cascade in response to specific extracellular stimuli, we examined the effects of environmental stresses and proinflammatory cytokines on activation of MKK4 and MKK7 protein kinase activities. We found that MKK4 and MKK7 isoforms were selectively regulated by upstream kinases (Fig. 7 and 8). Therefore, we examined the effects of UV-C radiation, anisomycin, TNF-α, and IL-1α on the regulation of MKK7 and MKK4 isoforms. The MKK4 and MKK7 isoforms were isolated, and their activity was measured by a coupled kinase assay. TNFα and IL-1α selectively activated the MKK7 isoforms and only weakly activated MKK4 (Fig. 9). In contrast, UV-C radiation and anisomycin caused selective activation of MKK4 compared to MKK7 (Fig. 9). These data indicate that the MKK4 and MKK7 isoforms are selectively regulated by specific extracellular stimuli. This selective regulation is consistent with the genetic evidence for nonredundant roles of MKK4 and MKK7 in Drosophila and mammals (38).

Subcellular localization of MKK4 and MKK7 protein kinases. JNK has been reported to accumulate in the nucleus upon treatment of cells with stimuli known to activate the JNK signaling pathway (8, 40, 51). We therefore examined the subcellular localization of MKK4 and MKK7 by immunofluorescence analysis. These studies demonstrated that the endoge-
nous MKK4 and MKK7 protein kinases were distributed in both the cytoplasm and the nucleus (Fig. 10A). Exposure of the cells to UV-C radiation or treatment with IL-1α did not cause marked changes in the distribution of the endogenous MKK4 or MKK7 protein kinases.

To examine the subcellular localization of individual MKK4 and MKK7 isoforms, we transfected cells with epitope-tagged MKK4 and MKK7 and also performed control experiments with epitope-tagged wild-type MEK1 and constitutively activated MEK1 (D_{N3}-MEK1-EE). The activated MEK1 was constructed by replacing the two activating phosphorylation sites with glutamic acid residues and by deletion of the NH₂-terminal NES (22, 23, 39, 47). Immunofluorescence analysis demonstrated that, as expected, wild-type MEK1 was restricted to the cytoplasm, while activated MEK1 was present in the nucleus (Fig. 10B). Under the same experimental conditions, the MKK4 and MKK7 isoforms were detected at low levels in the cytoplasm and appeared to preferentially accumulate in the nucleus (Fig. 10B). The higher level of nuclear accumulation of the transfected MAPKK than of the endogenous MAPKK may reflect the overexpression of the recombinant proteins. Exposure of the cells to UV-C radiation or treatment with IL-1α did not induce changes in the localization of MKK4 or MKK7 (data not shown).

Effect of nuclear exclusion on the activity of MKK7. The distribution of the MKK7 protein in the cytoplasm and the nucleus differs from that described for the ERK MAPK activator MEK1. While the endogenous MKK7 protein appears to preferentially accumulate in the nucleus (Fig. 10A), MEK1 is excluded from the nucleus. The mechanism of nuclear exclusion is mediated by the presence of an NES in the NH₂-terminal region of MEK1 (23). Disruption of the NES causes nuclear accumulation of MEK1 and marked potentiation of the activity of MKK7.

FIG. 8. Activation of MKK4 and MKK7 protein kinases by STE20 homologs. To examine the abilities of KHS and HPK1 to activate MKK7 isoforms, COS-7 cells were cotransfected with Flag-tagged MKK7α1, MKK7α2, MKK7β1, MKK7β2, MKK4, or MKK4β together with an empty expression vector (Control) or an expression vector for either KHS or HPK1. Protein expression was monitored by immunoblot (IB) analysis. Flag-tagged MKKs were immunoprecipitated, and MKK activity was measured in the immune complex by a coupled protein kinase assay (KA) using recombinant JNK1 and c-Jun as substrates. The phosphorylated c-Jun was detected after SDS-PAGE by autoradiography and quantitated by PhosphorImager analysis. MAPKK activity is presented as relative protein kinase activity. The levels of MAPKK activation caused by KHS and HPK were 15- and 31-fold (MKK7α1); 3.9- and 15-fold (MKK7α2); 4.0- and 3.5-fold (MKK7β1); 5.4- and 4.4-fold (MKK7β2); 3.2- and 2.9-fold (MKK4); and 4.4- and 2.4-fold (MKK4β), respectively. Similar results were obtained in three separate experiments.

FIG. 9. Regulation of MKK4 and MKK7 protein kinases by extracellular stimuli. 293 cells were transiently transfected with expression vectors (pEBG) encoding either MKK7α1, MKK7α2, MKK7β1, MKK7β2, MKK4, or MKK4β; 36 h after transfection, the cells were untreated (Control) or treated with UV-C (UV; 80 J/m²), anisomycin (ANISO; 5 μg/ml), TNF-α (20 ng/ml), or IL-1α (2 ng/ml). The cells were harvested after incubation at 37°C (30 min). MKK4 and MKK7 were isolated by using GSH-Sepharose beads, and the MKK activity was measured by a coupled protein kinase assay using recombinant JNK1 and c-Jun as substrates. The radioactivity incorporated into c-Jun was quantitated after SDS-PAGE by PhosphorImager analysis. MAPKK activity is presented as relative protein kinase activity. The levels of MAPKK activation caused by UV, anisomycin, TNF-α, and IL-1α were 1.4-, 1.5-, 2.2-, and 1.9-fold (MKK7α1); 2.8-, 1.5-, 3.9-, and 2.5-fold (MKK7α2); 2.4-, 2.1-, 2.5-, and 4.1-fold (MKK7β1); 2.0-, 1.7-, 2.8-, and 2.9-fold (MKK7β2); 4.7-, 3.2-, 0.9-, and 0.9-fold (MKK4); and 2.6-, 2.3-, 0.9-, and 0.9-fold (MKK4β), respectively. Similar results were obtained in three separate experiments.
MKK7 ENCODES A GROUP OF JNK KINASES

The genes that encode the JNK activators MKK4 and MKK7 are located on mouse chromosome 11. The MKK7 gene was mapped to mouse chromosome 11 (Fig. 2). It is interesting that the MKK4 gene is also located on this chromosome (81). The localization of both of the genes that encode JNK activators on the same mouse chromosome suggests that these genes might be linked. This is an interesting possibility because MKK4 is a candidate tumor suppressor gene that is mutated in certain tumors (73), suggesting that the MKK7 gene may also be a target of genetic alterations in disease processes.

Although the mouse MKK4 and MKK7 genes reside on the same chromosome, it does not appear that this relationship is present in all species. Thus, in Drosophila, the D-MKK4 (29) and D-MKK7 (26) genes are located on different chromosomes. Indeed, it appears that the D-MKK3 gene (and not the D-MKK4 gene) is tightly linked to the D-MKK7 gene in the fly (29). The colocalization of the genes that encode the JNK activators MKK4 and MKK7 on one mouse chromosome is therefore not evolutionarily conserved.

The MKK7 gene encodes a group of protein kinases. The MKK7 gene includes 14 exons. Alternative splicing leads to the inclusion or exclusion of exons located in the 5′ and 3′ regions of the gene, resulting in the expression of a group of MKK7 isoforms that differ in their NH2- and COOH-terminal regions (Fig. 3). The MKK7 protein kinase isoforms act as activators of JNK MAPK. Comparative studies demonstrate that the MKK7 isoforms differ in the extent of activation in response to different upstream components of the JNK signaling pathway (Fig. 7 to 9).

Sequences in the NH2 termini of MAPKk protein kinases have been reported to mediate specific interactions with other components of MAPK pathways (4). Similarly, specificity determinants have been identified in the NH2- and COOH-terminal regions of MAPK pathway components, including PBS2 and MEKK1 (60, 69, 85). It is therefore interesting that multiple cDNA clones with different 5′ regions have been reported for MKK3, MKK4, MKK5, and MKK6 (13, 17, 28, 54, 61, 65, 90). Whether these different cDNA clones correspond to fully processed mRNA is unclear because the corresponding genes have not been characterized. In contrast, it is established that the usage of alternative exons within the MKK7 gene leads to the formation of multiple protein kinase isoforms (Fig. 3). It is therefore likely that the expression of a group of alternatively spliced isoforms is a common property of MAPKk genes. The possible role of the divergent NH2-terminal sequences of MAPKk isoforms as specificity determinants for MAPKk function (4) suggests that different MAPKk isoforms may have different physiological functions. In this study, we demonstrate that the NH2-terminal extension that is present in the MKK7 isoforms, but not MKK7α isoforms, binds to the substrate JNK and may account, in part, for the differences in activity between these MKK7 isoforms (Fig. 4 and 5). This binding to JNK is consistent with the presence of consensus primary sequence motifs for JNK interaction (Leu-Xaa-Leu) in the NH2-terminal region of MKK7 (88).

The MKK7 group of protein kinases are present in the cytoplasm and the nucleus. Studies of the ERK MAPK signaling pathway demonstrate that the ERK MAPKs are present in the cytoplasm of quiescent cells and that the ERKs accumulate in the nucleus following activation (9, 27, 66). Similarly, it has been reported by several groups that JNK accumulates in the nucleus following activation (8, 40, 51). In contrast to the activation-induced nuclear accumulation of the ERK MAPKs, the ERK activators MEK1 and MEK2 are cytoplasmic enzymes (1). A nuclear export sequence in the NH2-terminal region of MEK1 appears to mediate rapid export out of the nucleus and, therefore, the accumulation of MEK1 in the cytoplasm (22, 23, 39). In contrast, we found that the JNK activator MKK7 was present in both the cytoplasm and the nucleus (Fig. 10). This nuclear location was observed for endogenous MKK7 (Fig. 10A) and for multiple MKK7 isoforms (Fig. 10B). Nuclear localization was also observed for the JNK activator MKK4. The mechanism that accounts for the nuclear location of MKK4 and MKK7 is unclear because the sequences of these...
protein kinases do not include an obvious nuclear localization sequence. It is possible that the absence of an NES may contribute to the localization of MKK4 and MKK7 in the nucleus. Thus, the subcellular location of the JNK activators MKK4 and MKK7 differs from that of the ERK activators MEK1 and MEK2.

Recent studies indicate that the subcellular organization of the p38 MAPK pathway differs from that of both the ERK and JNK signaling pathways. The major p38 MAPK activators (MKK3 and MKK6), like the activators of ERK (MEK1 and MEK2), are both preferentially located in the cytoplasm (5). However, unlike ERK, which exhibits activation-induced redistribution from the cytoplasm to the nucleus, the p38 MAPK is prelocalized in the nucleus and is rapidly exported to the cytoplasm upon activation (5). The mechanism of nuclear export appears to be mediated, at least in part, by the nuclear export signal (NES) of MEK1 (residues 32 to 51) was inserted following the Flag epitope to create NES-MKK7β. The expression of MKK7 was examined by immunoblot (IB) analysis using the Flag-specific monoclonal antibody M2 and detected with secondary antibodies conjugated to Texas red and fluorescein, respectively. DNA was visualized by staining with DAPI (blue). The scale bar (white) represents 10 μm. (C) Regulation of MKK7β2 and NES-MKK7β2 by extracellular stimuli. COS-7 cells expressing MKK7β2 or NES-MKK7β2 were untreated (Control) or treated (30 min) with UV-C (80 J/m²) or IL-1α (10 ng/ml). The expression of MKK7 was quantitated by PhosphorImager analysis. MAPKK activity is presented as relative protein kinase activity. The levels of MAPKK activation caused by UV and IL-1α were 3.0- and 3.7-fold (MKK7β2) and 1.5- and 1.8-fold (NES-MKK7β2), respectively. (D) Activation of JNK1 by MKK7β1 and NES-MKK7β2. COS-7 cells were cotransfected with plasmids expressing HA-tagged JNK1 together with an empty vector (Control) or an expression vector encoding Flag-tagged MKK7β1, NES-MKK7β1, MKK7β2, or NES-MKK7β2. The expression of MKK7 and JNK1 was monitored by immunoblot (IB) analysis using antibodies to Flag and HA, respectively. JNK1 activity was measured by immune complex kinase assay (KA) using the substrate c-Jun. The radioactivity incorporated into c-Jun was quantitated after SDS-PAGE by PhosphorImager analysis. JNK activity is presented as relative protein kinase activity. The levels of JNK activation observed for MKK7β1, NES-MKK7β1, MKK7β2, and NES-MKK7β2 were 21-, 27-, 25-, and 31-fold, respectively.
substrate MAPKAP kinase 2, which is also exported from the nucleus following activation (5, 16). Together, these data indicate that there are marked differences between the subcellular organization of the ERK, JNK, and p38 MAPK pathways.

The presence of MKK4 and MKK7 in the nucleus suggests that these MAPKK may activate JNK in this compartment of the cell. Indeed, Mizukami et al. (51) have recently reported that JNK may be activated in the nucleus during ischemia-reperfusion injury to the heart. However, it is possible that MKK4 and MKK7 may also activate JNK in the cytoplasm. Evidence in favor of this hypothesis was obtained from studies of mutated MKK7 protein kinases that are excluded from the nucleus (Fig. 11). We found that cytoplasmic MKK7 protein kinases efficiently activated JNK in vivo (Fig. 11D) and that the activated JNK in these cells accumulated in the nucleus (Fig. 11B). These data indicate that MKK7 (and possibly MKK4) can activate JNK in the cytoplasm and that the activated JNK redistributes from the cytoplasm to the nucleus.

The presence of MKK4 and MKK7 in the nuclei of quiescent and stimulated cells indicates that these MAPK isoforms may also be activated in the nucleus. Fanger et al. have reported that MEK1 is located in the nucleus (20). However, other MAPKKK that activate the JNK signaling pathway appear to be preferentially located in the cytoplasm. For example, MLK2 is detected in punctate structures along microtubules (56), MEKK2 and MEKK3 are located on Golgi-associated vesicles (20), while MLK3 and DLK were identified in the cytoplasm by immunofluorescence microscopy (data not shown). The cytoplasmic location of these MAPKKKs raises questions concerning the mechanism of action of nuclear MKK4 and MKK7. Clearly MAPKKKs and MAPKKs must interact and therefore have to be present in the same cellular compartment. The simplest explanation is that although MKK4 and MKK7 are present in the nucleus, a cytoplasmic population of molecules may arise from rapid shuttling across the nuclear membrane. This model implies that some MAPKKs (e.g., MEK1) may directly activate MKK4 and MKK7 in the nucleus, whereas others (e.g., MLK3) may activate a cytoplasmic population of MKK4 and MKK7 that rapidly shuttles to the nucleus.

Further studies are required to identify the mechanisms used to determine the subcellular distribution of components of the JNK signaling pathway. Such mechanisms may include scaffold proteins that interact with JNK and MKK7 (82). The expression of scaffold proteins may contribute to the selective control of JNK signaling in specialized differentiated cells.

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