Human Mitogen-Activated Protein Kinase Kinase 7 (MKK7) Is a Highly Conserved c-Jun N-terminal Kinase/Stress-activated Protein Kinase (JNK/SAPK) Activated by Environmental Stresses and Physiological Stimuli*

(Received for publication, September 29, 1997, and in revised form, January 21, 1998)

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We report the cloning of a novel human activator of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase kinase 7 (MKK7). The mRNA for MKK7 is widely expressed in humans and mice and encodes a 47-kDa protein (419 amino acids), as determined by immunoblotting endogenous MKK7 with an antibody raised against its N terminus. The kinase domain of MKK7 is closely related to a Drosophila JNK kinase dHep (69% identity) and to a newly identified ortholog from Caenorhabditis elegans (54% identity), and was more distantly related to MKK4, MKK3, and MKK6. MKK7 phosphorylated and activated JNK1 but failed to activate p38 MAPK in co-expression studies. In hematopoietic cells, endogenous MKK7 was activated by treatment with the growth factor interleukin-3 (but not interleukin-4), or by ligation of CD40, the B-cell antigen receptor, or the receptor for the Fc fragment of immunoglobulin. MKK7 was also activated when cells were exposed to heat, UV irradiation, anisomycin, hyperosmolarity or the pro-inflammatory cytokine tumor necrosis factor-α. Co-expression of constitutively active mutants of RAS, RAC, or CDC42 in HaCaT epithelial cells or of RAC or CDC42 in Ba/F3 factor-dependent hematopoietic cells also activated MKK7, suggesting that MKK7 will be involved in many physiological pathways.

The responses of eukaryotic cells to extracellular stimuli are remarkably well conserved in evolution. In mammalian cells, most extracellular stimuli result in the activation of three families of Ser/Thr protein kinases, the mitogen-activated protein kinases (MAPK)1 (1, 2). The extracellular-regulated kinases (ERK) MAPK are activated in response to growth factors including EGF and hematopoietic growth factors (1, 3). Members of the other two major families of MAP kinases, the c-Jun N-terminal kinases (JNK) or stress-activated protein kinases (SAPK) and the p38 MAP kinases, are strongly activated in response to stresses such as heat, UV light, anisomycin, or hyperosmolarity (4–8). However, recent work has shown that members of both families of stress-activated protein kinases are also activated by hematopoietic growth factors such as IL-3, GM-CSF, Steel locus factor, erythropoietin, granulocyte colony-forming factor (9–13), as well as by ligation of immunoregulatory molecules such as the antigen receptors of B and T lymphocytes, the Fc receptors of myeloid cells, CD40 or Fas (14–18).

MAP kinases are activated by dual specificity MAP kinases (MKK) through phosphorylation of the threonine and tyrosine residues of a TXY motif present in their activation loops (subdomain VIII). Kinases of the JNK/SAPK family are characterized by a TPY activation motif (5, 6) and are activated by an MKK termed MKK4 or SEK1 (19–21). Several lines of evidence point to the existence of another MKK capable of activating the JNK/SAPK family. Moriguchi et al. (22) identified at least one JNK/SAPK kinase activity in addition to the activity of MKK4 in rat fibroblastic 3Y1 cells stimulated by hyperosmotic shock. Similarly Meier et al. (23) identified two JNK/SAPK kinase activities operationally termed SAPK kinase-4 and SAPK kinase-5 in KB cells, a human oral carcinoma cell line, following treatment with hyperosmolarity or anisomycin. The generation of embryonic stem (ES) cells in which the mkk4 gene was disrupted provided conclusive evidence for the existence of another JNK kinase, as ES cells lacking MKK4 still exhibited increased JNK activity following treatment with hyperosmolarity or UV irradiation (24, 25).

We now report the molecular cloning and characterization of a novel human MKK (MKK7) and the identification of its ortholog in Caenorhabditis elegans (cMKK7). The mRNA for MKK7 was widely expressed and encodes a 419-amino acid protein that is most highly related to the Drosophila MKK known as dHep (26). MKK7 was capable of phosphorylating and activating JNK1 both in vivo and in vitro but was unable to activate p38 MAPK in vivo. MKK7 was activated in response to the hematopoietic growth factor interleukin-3 (IL-3) or ligation of three receptors involved in immune regulation as follows: CD40, the receptor for the Fc fragment of immunoglobulin G (FcR), or the B-cell antigen receptor (BCR), as well as by stresses such as hyperosmolarity, heat shock, UV irradiation, anisomycin, or the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α). Co-expression of MKK7 with constitutively active mutants of the small GTPases Ras, Rac, or CDC42 indicated that MKK7 was downstream of Ras, Rac, or

* This work was supported in part by the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) hMKK7a, AF013588; hMKK7b, AF013589; mMKK7a, AF022112; and mMKK7b, AF022113.

‡ Supported by a Medical Research Council Studentship.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; IL, interleukin; TNF-α, tumor necrosis factor-α; FcR, Fc receptor for IgG; BCR, B-cell antigen receptor; MAP, mitogen-activated protein; MKK, MAP kinase kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ATF-2, activating transcription factor-2; GST, glutathione S-transferase; ES, embryonic stem; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PCR, polymerase chain reaction; bp, base pair(s); aa, amino acid(s); EST, expressed-sequence tag; G-CSF, granulocyte colony-stimulating factor.
Molecular Cloning of MKK7—An expressed sequence tag (EST) clone (aa 019720) containing the 3′ end (464 bp) of MKK7 was identified by screening the EST data base (University of Washington-Merck EST project) with a degenerate oligonucleotide sequence based on homology between other MKK (aa FVX3PXHDXLXKX3RXH). We used oligonucleotide primers based on this MKK7 sequence to amplify the 5′ end of the kinase from a human fetal kidney rapid amplification of cDNA ends cDNA library using Vent DNA polymerase for polymerase chain reaction (PCR). All PCR products were subcloned, and all colonies were transferred onto nitrocellulose and Southern blotted with the EST fragment that had been radiolabeled by random priming. We used this technique to obtain a further 861 bp of MKK7 that contained an in-frame stop codon and a splice variant of MKK7 (hMKK7β). The cDNA encoding full-length MKK7α was cloned from at least two separate PCR reactions and was sequenced on both strands. We used degenerate primers based on the human sequence to amplify the murine cDNA from Ba/F3 hematopoietic cell cDNA. The EST data base was screened to identify a different splice variant of murine MKK7 (mMKK7β). Published data bases were screened for MKK related to MKK7 using the National Center for Biotechnology Information Advanced BLAST search program.

cDNA Constructs—Human MKK7 was amplified by PCR using primers to add an in-frame BamHI site to its 5′ end to allow subcloning into pEFBOS-Nxyr3 and pEG vectors. The vectors encoding pEG-JNK1 and pEG-SEK1 were the gifts of Dr. L. Zon. The mammalian expression vector encoding Flag-tagged CSBP2/m8 MAPK was given by Dr. Peter Young. The vectors encoding constitutively active RAS61V, RACVal-12, and CDC42 Val-12 were received from Dr. R. Kay, Dr. F. McCormick, and Dr. R. Cerione, respectively. The prokaryotic expression vector for GST-JNK1 was produced from pEPG-JNK1 by restriction digestion with BamHI and EcoRI. The expression vector for GST-JNK1 was then subcloned into the polynucleotide of pGEX-5×. Recombinant Proteins and Antibodies—GST-JNK1 and GST-c-Jun (1–169) were expressed in Echerichia coli (UT56000 strain) and were purified by affinity chromatography on glutathione-Sepharose beads (Amersham Pharmacia Biotech). The anti-p38 MAP kinase polyclonal antibody used for Western blotting (sc-535), the anti-MKK4 polyclonal antibody (sc-964), and ATF-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody used to cross-link CD40 was a gift of Dr. M. Howard, and the sheep anti-rat Ig antibody (a-g) were purchased from PharMingen (San Diego, CA), and the polyclonal rabbit anti-rat Ig antibody (a-g) used for cross-linking. All other antibodies are the same as in our previous publication. All samples were immunoprecipitated using the rabbit polyclonal antibody raised against the N terminus of MKK7, and endogenous MKK4 was immunoprecipitated with an a-MKK4 antibody. Flag-tagged p38 MAPK was immunoprecipitated using the M2 antibody and protein G-Sepharose.

Protein Kinase Assays—Beads with associated kinases were washed extensively with solubilization buffer and once with kinase assay buffer (25 mM HEPES, pH 7.5, 8 mM magnesium chloride, 2 mM dithiothreitol, 50 mM β-glycerol phosphate, and 0.5 mM sodium vanadate). MKK7 activity was measured directly by the ability of MKK7 to phosphorylate 1 μg of GST-JNK1 in the presence of exogenous free GST protein to prevent the substrate from binding to the beads. MKK7 activity was also assayed indirectly by first incubating MKK7 with 1 μg of GST-JNK1 in the presence of unlabelled ATP for 30 min and then removing the aliquot and determining the activity of GST-JNK1 in an in vitro kinase reaction using 1 μg of GST-c-Jun as substrate. All kinase reactions were initiated by the addition of kinase assay buffer containing the appropriate substrate and 10 μCi of [γ-32P]ATP and stopped after 20 min by the addition of SDS-sample buffer. For p38 MAP kinase assays, Flag-tagged p38 MAPK was immunoprecipitated using a-Flag antiserum and protein G-Sepharose beads. The beads were washed and kinase activity was assessed using ATF-2 as a substrate. The only modification was the inclusion of an additional unlabeled ATP (50 μM).

RESULTS

Molecular Cloning of the cDNA of MKK7—We screened the EST (expressed sequence tags) data base using a degenerate cDNA encoding full-length MKK7α present in the cDNA of both human and murine both were fused at their N termini to the T-cell epitope from tetanus toxin (aa 1–10). The recombinant expressed proteins were used to immunize rabbits and the resulting antisera and protein G-Sepharose beads. The beads were washed and kinase activity was assessed using ATF-2 as a substrate. The only modification was the inclusion of an additional unlabeled ATP (50 μM). Phosphorylated proteins were visualized after SDS-PAGE by autoradiography. Densitometry was performed using an HSI scanning densitometer. The MKK from C. elegans (CMKK7) was previously submitted to GenBank® as part of the C. elegans genome sequencing project (28) and was given the accession number U38377 (gene k08a8.1).
oligonucleotide sequence based on the amino acids conserved in the C terminus of all previously characterized mitogen-activated protein kinase kinases (19). We identified 10 clones that encoded known MKK family members and one clone that contained a novel human sequence. This clone contained 464 base pairs (bp) of coding region and approximately 1200 bp of 3'-untranslated region. We used 5'-rapid amplification of cDNA ends to isolate overlapping 5'-fragments of this novel cDNA from a human fetal kidney rapid amplification of cDNA ends cDNA library (CLONTECH). By using this technique we cloned a further 861 bp of human MKK7 (hMKK7a) that contained an in-frame stop codon. When translation was initiated at the first methionine, this cDNA encoded a 419-amino acid protein (Fig. 1A) that contained a putative protein kinase domain. We also isolated a splice variant of human MKK7 (MKK7b) that contained an additional 126 base pairs encoding an insert of 42 amino acids (Fig. 1A). We screened the yeast and invertebrate data bases for other MKK related to MKK7, and although we failed to identify a yeast homolog, we did find an ortholog of MKK7 in Drosophila melanogaster (dHep, 69% identity) (26). In C. elegans we identified an ortholog with 54% identity we termed cMKK7 (28). As seen in the radial dendrogram comparing the catalytic domains of MKK7 with dHep, cMKK7, and all other known human MKK (Fig. 1B), MKK7 was orthologous with dHep and cMKK7 and was more closely related to these than to the known human MKK.

We used primers derived from the human sequence of MKK7 to amplify an 869-bp fragment of cDNA from the murine Ba/F3 hematopoietic cell line. Sequencing indicated that this cDNA encoded the N terminus and most of the kinase domain of a murine homolog of MKK7 (mMKK7a). We screened the EST data base for cDNA encoding the N terminus and identified another splice variant of mMKK7 (mMKK7g) that contained an additional 48-base pair exon or in-frame intron (Fig. 1A). We have not yet identified a human homolog of mMKK7g. These splice variants may encode alternative forms of MKK7 or may represent incompletely processed mRNA. By far the most highly represented form of MKK7 in a human fetal kidney cDNA library was MKK7a, and therefore we chose this isoform for continued analysis of MKK7 function.

Expression and Tissue Distribution of MKK7—To investigate expression of endogenous and exogenous MKK7, we immunoblotted lysates of Ba/F3 and MC/9 hematopoietic cells and endogenous and exogenous MKK7 in lysates of a clone of Ba/F3 cells that expressed human myc-tagged MKK7. The indicated numbers of cells were lysed, and proteins were separated by SDS-PAGE. An antisera raised against the N terminus of MKK7 (aa 4–26) was used for immunoblotting, and immunoreactive proteins were detected by enhanced chemiluminescence. The position of endogenous MKK7 and myc-tagged exogenous MKK7 are indicated by arrowsheads. B, Northern blot of MKK7 mRNA using poly(A)+ mRNA isolated from multiple human tissues.

![Fig. 1. Primary structure and phylogenetic relationships of MKK7 protein kinases.](image1)

![Fig. 2. Expression and tissue distribution of MKK7.](image2)
predicted by translation of the cDNA encoding mMKK7α or mMKK7γ. We were unable to detect an immunoreactive protein at the size (53 kDa) predicted to correspond to the hMKK7β splice variant in murine cells. These experiments indicated that the levels of exogenous MKK7 expressed in Ba/F3-MKK7 cells were at least 30-fold greater than endogenous MKK7 (Fig. 2A). This was interesting as the Ba/F3-MKK7 cells exhibited no obvious abnormalities in growth and remained dependent on IL-3 for growth and survival. Moreover, overexpression of human MKK7 did not appear to affect the expression of endogenous murine MKK7, as 5 × 10⁶ cells of both untransfected and Ba/F3-MKK7 cells expressed comparable levels of endogenous MKK7 (Fig. 2A).

We used Northern blotting to investigate the expression of MKK7 in multiple human tissues. The probe contained the unique N terminus of MKK7 and the 5’-end of the kinase domain which exhibits little sequence homology with other known MKK family members. The probe bound to a single transcript of around 4 kilobase pairs in all tissues tested, with highest levels of hybridization occurring with mRNA from skeletal muscle (Fig. 2B). We also used reverse transcriptase-PCR to examine expression of MKK7 in a number of human and murine cells and confirmed the PCR products as derived from MKK7 by sequencing and restriction analysis. MKK7 mRNA was present in all tested cell lines corresponding to a number of cell lineages (Table I). Thus, MKK7 was widely expressed.

**Activation of JNK1, but Not p38, by MKK7—**It has been previously shown that the transient co-expression of mitogen-activated protein kinases with the appropriate upstream MKK leads to *in vivo* activation of the MAPK. We investigated the substrate specificity of MKK7 by co-expression of MKK7 with either JNK1 or p38 MAP kinase. Co-expression of MKK7 with JNK1 in HeLa epithelial cells (Fig. 3A) or Ba/F3 hematopoietic cells (data not shown) without any deliberate stimulation of the cells resulted in easily detectable activation of JNK1. Stimulation of these co-transfected cells with hyperosmotic shock (0.2 M NaCl) or UV light resulted in even greater JNK1 activation (Fig. 3A). These results suggested that MKK7 was upstream of JNK1 and was activated by stimulation of cells by UV light or hyperosmotic shock. In contrast, co-expression of p38 MAPK with MKK7 had no effect on activation of p38 MAPK (Fig. 3B). Nor did co-expression of MKK7 with p38 MAPK result in increased activation of p38 MAPK when the transfected cells were stimulated by hyperosmotic shock or UV light. Thus MKK7, unlike MKK4, specifically activated JNK1 but not p38 MAP kinase.

**Transiently Expressed MKK7 Is Activated in Response to Multiple Stimuli—**The c-Jun N-terminal kinases have been shown to be activated in response to a number of different stimuli including UV light, hyperosmotic shock, protein synthesis inhibitors, heat shock, the pro-inflammatory cytokines IL-1 and TNF-α, and hematopoietic growth factors (4, 10). We tested the ability of these stimuli to activate GST-MKK7 that had been transiently overexpressed in HeLa or Ba/F3 cells. GST or GST-MKK7 was purified from extracts of cells subjected to various stimuli, and its ability to phosphorylate GST-JNK1 was assayed in an *in vitro* kinase assay. As shown in Fig. 4A, the activity of MKK7 was increased by treatment of cells with hyperosmotic shock (11-fold), heat shock (7.5-fold), UV light (5.5-fold), or TNF-α (3.5-fold) but not by EGF (Fig. 4A). The ability of these stimuli to activate MKK7 and to in turn activate JNK1 *in vitro* was also assessed. We observed the ability of MKK7 to activate JNK1 was increased in transfected cells treated with TNF-α (4-fold), hyperosmotic shock (5-fold), or antisense (5-fold for 30 min; 6.5-fold for 45 min) (Fig. 4B). Thus MKK7 was activated by all these known activators of JNK. We and others (10–12) have previously demonstrated the activation of JNK in response to the hematopoietic growth factor IL-3 in both Ba/F3 hematopoietic cells and MC/9 mast cells. In contrast, the related hematopoietic growth factor IL-4 failed to activate JNK (10). We transiently expressed GST-MKK7 in Ba/F3 cells, subjected them to various stimuli, and assayed the ability of MKK7, which was activated *in vivo*, to phosphorylate GST-JNK1 in *vitro*. As seen in Fig. 4C, MKK7 was strongly activated compared with the activity of MKK7 from unstimulated cells, in cells treated with IL-3 (5.5-fold), 0.2 M NaCl (4.5-fold), or UV light (3.5-fold). In contrast, MKK7 was not activated in cells treated with IL-4 (Fig. 4C), correlating with its inability to activate JNK (10).

**Activation of Endogenous MKK4 and MKK7 by Various Physiological Stimuli—**We next investigated the activation of endogenous MKK7 in response to physiological stimuli. In parallel we also investigated the activation of the known JNK activator MKK4 to identify potential functional differences. The antibodies used for immune complex kinase assays specifically recognized MKK4 or MKK7 (Fig. 5A). We first examined the ability of TNF-α to activate MKK4 and MKK7 in HeLa cells. As seen in Fig. 5B, we observed an increase in the activity of both MKK4 (5-fold) and MKK7 (2.5-fold). We then examined the activity of IL-3, IL-4, antisense, and 0.2 M NaCl to activate MKK7 in Ba/F3 cells. Consistent with the results of experiments with transiently expressed MKK7, cells treated with

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**Table I**

| Cell line | Species      | Cell lineage               |
|-----------|--------------|----------------------------|
| HeLa      | Human Epithelial (cervical carcinoma) |
| HepG2     | Human Hepatocarcinoma |
| Jurkat    | Human Acute T-cell leukemia |
| Daudi     | Human B lymphoma |
| HEK 293   | Human Embryonic kidney cell |
| U937      | Human Monocytic |
| 129/J ES cells | Mouse Embryonic stem cell |
| Ba/F3     | Mouse IL-3-dependent hematopoietic cell |
| MC/9      | Mouse IL-3-dependent mast cell |

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**Fig. 3.** Co-expressed MKK7 activates JNK1 but not p38 MAPK.

HeLa cells were transfected with constructs encoding GST-JNK1 (A) or Flag-tagged CSBP2/p38 (B), together in each case with a construct encoding myc-tagged MKK7 or as a control the empty vector pEF-BOS (pEF). Transfected cells were split into three plates and either left untreated (Con) or stimulated with 0.2 M NaCl (Na) or UV irradiation (UV) for 20 min. GST-JNK1 was affinity puriﬁed with glutathione-Sepharose, and its kinase activity was determined using GST-c-Jun as substrate. Flag-tagged p38 MAPK was immunoprecipitated with the M2 antibody, and its activity was determined using ATG-2 as substrate. The phosphorylation of substrates was visualized after SDS-PAGE using autoradiography.
MKK7 Is Activated by Physiological and Environmental Stimuli

**Fig. 4. Activation of MKK7 by various stimuli.** Cells were transiently transfected with vectors encoding GST or GST-MKK7, and aliquots were stimulated as follows. A, HeLa cells were either left untreated (Con) or stimulated with 100 ng/ml EGF (EGF) for 5 min, 0.2 M NaCl (Na) for 20 min, UV irradiation (UV) for 20 min, 100 ng/ml TNF-α (TNF) for 20 min, or heat-shocked (Heat) at 42°C for 20 min. B, HeLa cells were left untreated (Con) or stimulated with 100 ng/ml TNF-α (TNF) for 15 min, 50 μg/ml anisomycin (Aniso) for 30 or 45 min, or 0.2 M NaCl (Na) for 20 min. C, Ba/F3 cells were left untreated (Con) or stimulated with 10 μg/ml synthetic IL-3 (IL-3) for 5 min, 10 μg/ml synthetic IL-4 (IL-4) for 10 min, 0.2 M NaCl (Na) for 20 min, or UV irradiation (UV) for 20 min. In all cases, transiently expressed proteins were affinity precipitated using glutathione-Sepharose from samples of lysates that had been normalized for total protein using the Pierce assay. MKK7 activity was determined by assaying its ability to phosphorylate GST-c-Jun as a readout of JNK activity. We also performed similar experiments in the murine hematopoietic cell line Ba/F3. Co-expression of GST-JNK1 and the constitutively active mutants of RAC and CDC42 resulted in activation of JNK1 in Ba/F3 cells; however, co-expression of activated RAS with JNK1 resulted in little or no activation of JNK1 (data not shown). In keeping with these findings, co-expression of MKK7 and RAC or CDC42 and MKK7 in HeLa cells resulted in marked activation of MKK7, demonstrating that this kinase could be activated by signals downstream of each of these GTPases (Fig. 6A). Similar results were obtained when GST-MKK4 was co-expressed with these GTPases in HeLa cells (Fig. 6A). We also performed similar experiments in the murine hematopoietic cell line Ba/F3. Co-expression of GST-JNK1 and the constitutively active mutants of RAS and CDC42 activated JNK in Ba/F3 cells; however, co-expression of activated RAS with JNK1 resulted in little or no activation of JNK1 (data not shown). In keeping with these findings, co-expression of MKK7 and RAC or CDC42 in Ba/F3 cells activated MKK7; however, the co-expression of activated RAS with MKK7 was not sufficient to activate MKK7 (Fig. 6B).

**DISCUSSION**

Our results indicate that human and murine MKK7 are highly conserved (99% identity) and are most closely related to dHep, a MKK in *Drosophila*, exhibiting 69% identity in the kinase domain. MKK7 was less related to MKK4, MKK6, or MKK3 (45–55% identity), the other mammalian MKK known to be activators of JNK and p38 MAPK, and was even less similar to MEK1 or MEK2 (45–50% identity). We conducted extensive searches of the yeast and invertebrate data bases, and although we failed to identify MKK7 in *Saccharomyces cerevisiae*, we did identify a highly related *C. elegans* MKK (cMKK7) that exhibited 54% identity to MKK7 within the kinase domain (28). A more detailed inspection of alignments revealed a series of residues or motifs that were conserved among dHep, MKK7, and cMKK7 that were not present in MKK3, MKK4, and MKK6, and vice versa. Particularly striking are differences in the activation loops, with MKK7, dHep, and cMKK7 being characterized by a basic amino acid (S/K/R/AKT), at the same position where in MKK3, MKK4, and MKK6 a hydrophobic residue (S/I/V) is found. Overall these patterns of conservation of specific motifs support the notion that MKK7 is orthologous with dHep and cMKK7 and is more distantly related to MKK4, MKK3, and MKK6 (Fig. 1B). It will be interesting to determine the functional significance of the conserved residues that characterize MKK7, dHep, and cMKK7 and distinguish them from MKK4, MKK3, and MKK6.

Tournier et al. (32) have recently reported the cloning of two

IL-3 exhibited an increased activation (8-fold) of endogenous MKK7 (Fig. 5C). Cells treated with IL-3 also exhibited an increase in MKK4 activity (3-fold). In contrast, cells treated with IL-4 failed to activate MKK7 (Fig. 5C). Anisomycin and hyperosmotic shock also activated endogenous MKK7 (4- and 14-fold).

We then examined other known activators of JNK for their ability to activate MKK7. Previous studies in the immature B lymphoma WEHI-231 have shown that cross-linking the antigen receptor of B lymphocytes (BCR) weakly activates JNK, and ligation of CD40 greatly increases JNK activity (15, 16). We cross-linked either CD40 or the BCR using antibodies as described previously (18) and examined the activity of endogenous MKK4 and MKK7. We observed an increase in MKK7 activity upon cross-linking of either CD40 (9-fold) or the BCR (3.5-fold) in WEHI-231 cells (Fig. 5D). Similarly, MKK4 was activated by ligation of CD40 and very weakly by cross-linking of the BCR (Fig. 5D).

Activation of JNK has also been shown to follow cross-linking of the receptor for the Fc fragment of immunoglobulin G (FcR) on myeloid cells. The monoclonal antibody 2.4G2 (α-FcR) binds to both FcγRII and FcγRIII and when cross-linked by a secondary antibody (α-Ig) induces signaling through the FcR. As seen in Fig. 5E, cells either left untreated or incubated with either α-FcR or α-Ig alone exhibited base-line activities of MKK4 or MKK7. However, when cells were pretreated for 10 min with α-FcR and then incubated with α-Ig to aggregate the α-FcR-FcR complexes, an increase in both MKK4 (7-fold) and MKK7 (8-fold) activity was observed.

MKK7 Activates JNK Downstream of RAS, RAC, and CDC42—Expression of constitutively active mutants of the RAS and RHO family of small GTPases activates the JNK family of protein kinases (6, 7, 29–31). We confirmed that co-expression in HeLa cells of GST-JNK1 and RAS(Lys-61), RACVal-12, or CDC42Val-12 activated the JNK pathway (data not shown). We then co-expressed GST-MKK7 with each of these mutant GTPases, affinity-precipitated the GST-MKK7, and assessed its ability to activate GST-JNK1 in vitro, using phosphorylation of GST-c-Jun as a readout of JNK1 activity. We found that co-expression of activated mutants of RAS, RAC, or CDC42 and MKK7 in HeLa cells resulted in marked activation of MKK7, demonstrating that this kinase could be activated by signals downstream of each of these GTPases (Fig. 6A). Similar results were obtained when GST-MKK4 was co-expressed with these GTPases in HeLa cells (Fig. 6A). We also performed similar experiments in the murine hematopoietic cell line Ba/F3. Co-expression of GST-JNK1 and the constitutively active mutants of RAC and CDC42 resulted in activation of JNK1 in Ba/F3 cells; however, co-expression of activated RAS with JNK1 resulted in little or no activation of JNK1 (data not shown). In keeping with these findings, co-expression of MKK7 and RAC or CDC42 in Ba/F3 cells activated MKK7; however, the co-expression of activated RAS with MKK7 was not sufficient to activate MKK7 (Fig. 6B).
MKK7 Is Activated by Physiological and Environmental Stimuli

**Fig. 5. Activation of endogenous MKK7 by various physiological stimuli.** A, specificity of the antibodies used for immune complex kinase assays for MKK4 and MKK7. A lysate of WEHI-231 cells was split and immunoprecipitated with antibodies recognizing either MKK4, the N terminus of MKK7, or an irrelevant epitope (Con). Aliquots of immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotted with either an antibody recognizing MKK4 (left) or the C terminus of MKK7 (right). A sample containing $5 \times 10^5$ cell equivalents of whole cell extract (WCE) was analyzed in parallel. The position of MKK4 (two isoforms) and MKK7 are indicated by arrowheads. B, HeLa cells were left untreated (Con) or stimulated with 100 ng/ml TNF (TNF) for 5 or 10 min. C, Ba/F3 cells were left untreated (Con) or stimulated with 10 ng/ml synthetic IL-3 (IL-3) for 5 min, 10 μg/ml synthetic IL-4 (IL-4) for 10 min, 0.2 M NaCl (Na) for 20 min, or anisomycin (Aniso) for 30 min. D, WEHI-231 cells (2 x 10⁶) were left untreated (Con) or stimulated with 0.2 M NaCl (Na) for 20 min, 40 μg/ml α-IgM F(ab)₂ (α-IgM) to cross-link the antigen receptor of B lymphocytes (BCR) for 10 min, or 10 μg/ml IC10 (α-CD40) for 15 min. E, MC/9 mast cells were pretreated with α-FcR (α-FcR) for 10 min as indicated and then incubated for 10 min in medium alone or with a rabbit α-rat immunoglobulin antibody (α-Ig). B–E, cellular lysates were immunoprecipitated with antibodies recognizing MKK4 or MKK7. The activity of MKK4 or MKK7 was determined by incubating 1 μg of GST-JNK1 with 50 μg unlabeled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.

**Fig. 6. Activation of MKK7 by the small GTPases RAS, RAC, and CDC42.** HeLa (A) or Ba/F3 (B) cells were transiently co-transfected with a vector encoding GST, GST-MKK7, or GST-MKK4 and pEF-BOS that encoded the constitutively active mutants of RAS (Ras), RAC (Rac), or CDC42 (Cde42) or an empty vector as a control. In every case, a reporter construct encoding β-galactosidase in the same pEF-BOS vector was also co-transfected, and β-galactosidase activity was used to normalize the lysates for the expression of transfected genes. Cells transfected with the empty vector and MKK7 were left unstimulated (Con) or stimulated with 0.2 M NaCl (Na) for 20 min as controls for the kinase assay. The activity of affinity purified MKK7 was determined by incubating 1 μg of GST–JNK1 with 50 μg unlabeled ATP for 30 min and testing the ability of an aliquot of this reaction to phosphorylate 1 μg of GST-c-Jun. Phosphorylated proteins were visualized after SDS-PAGE by autoradiography.

splice variants of murine MKK7 and the N terminus of human MKK7α. The amino acid sequence predicted from their murine MKK7a clone is identical to the sequence that we report here, with the exception that it lacks the N terminus that was present in all of our predicted human and murine MKK7 splice variants. We were able to immunoblot endogenous MKK7 in lysates of Ba/F3 hematopoietic cells and MC/9 mast cells using an antiserum raised against the N terminus of our human and murine MKK7 sequence (Fig. 2A), and therefore we are confident that the form of MKK7 predicted from our human and murine cDNA is indeed expressed in murine cells.

Since submission of this manuscript there have been a number of articles describing the cloning of MKK7 (33–38). Holland et al. (33) have identified two isoforms of murine MKK7, one corresponding to murine MKK7α and the other with a unique N terminus. Moriguchi et al. (34) have cloned murine MKK7γ and identified two MKK7 isoforms using an antiserum raised against full-length murine MKK7γ. Based on electrophoretic mobility, we believe the larger isoform could represent the murine equivalent of our human MKK7β (Fig. 1A). Lu et al. (35) have identified another isoform of human MKK7 that has the same N terminus and kinase domain as our MKK7 isoforms but contains 70 amino acids that are unique to any of the MKK7 isoforms reported to date.

Based on sequence identities between the known MKK7 (31), we investigated the ability of MKK7 to activate JNK or p38 MAPK and showed that in co-transfection assays MKK7 acted upstream of JNK1 (3A) but not p38 MAPK (3B). The identification of JNK1 as an *in vitro* substrate for MKK7 permitted us to investigate the ability of a range of stimuli to activate transiently expressed MKK7. In HeLa cells, MKK7 was strongly activated by hyperosmotic shock, UV light, anisomycin, heat shock, and to a lesser extent TNF-α (Fig. 4, A and B). We observed no detectable activation of MKK7 in HeLa cells treated with EGF (Fig. 4A). These results are similar to that seen by Holland et al. (33) with platelet-derived growth factor in NIH3T3 cells. In Ba/F3 hematopoietic cells, hyperosmotic shock and UV light also activated MKK7 (Fig. 4C). Our observations that stimulation of Ba/F3 cells with IL-3 increased the activity of MKK7 (Figs. 4C and 5C) correlate with recent observations that JNK was activated by a range of hematopoietic growth factors including IL-3, granulocyte-macrophage colony-stimulating factor, G-CSF, erythropoietin or Steel locus factor (10–13). Moreover our observation that IL-4 failed to activate MKK7 (Figs. 4C and 5C) correlates with the inability of IL-4 to
activate the RAS, ERK, p38, or JNK MAP kinase pathways in hematopoietic cells (3, 9–10, 39–40).

Co-expression of activated mutants of RAS, RAC, or CDC42 with MKK7 in HeLa cells resulted in readily detectable activation of MKK7 and MKK4 (Fig. 6A). This is consistent with previous work that demonstrated that the RAS and RHO family of small GTPases are capable of activating JNK (6, 7, 29–31). Slightly different results were obtained in Ba/F3 cells where MKK7 was activated by co-expression of activated RAC or CDC42 but not RAS (Fig. 6B). The inability of RAS to activate MKK7 was not surprising as a constitutively active RAS was insufficient to activate JNK in Ba/F3 cells (11), although IL-3-induced JNK activation was blocked by expression of a dominant-negative mutant of RAS (11). Taken together, these results support the notion that RAS is necessary but not sufficient for JNK activation. Our evidence that IL-3 increased MKK7 activity (Figs. 4C and 5C), but that RAS alone failed to increase MKK7 activity (Fig. 6B), is also consistent with data on activation of JNK by G-CSF, which is structurally related to IL-3 and acts through a similar receptor. These experiments showed that activation of JNK in cells stimulated with G-CSF depended upon both an intact RAS signaling pathway as well as a specific tyrosine residue in the G-CSF receptor (13). Together these data suggest that activation of MKK7 in response to IL-3 involves both activation of the RAS pathway and an as yet unknown signal.

Depending on differentiation of the B lymphocyte and the presence of co-stimulatory molecules such as CD40, signaling through the BCR can initiate signals for either growth and survival or death of the B lymphocyte (41). Previous studies have shown the BCR to be a weak activator of JNK (16). We observed that ligition of the μ-chain of the BCR on WEHI-231 cells induced weak, but reproducible, activation of MKK7 (Fig. 5D), consistent with the activation of JNK we observed after ligation of the BCR (data not shown).

The receptor for the Fc fragment of immunoglobulin G (FcR) is structurally related to the BCR and has many important roles in the immune system (42). These include the phagocytosis of Ig-coated particles by macrophages and neutrophils, the antibody-dependent cell-mediated cytotoxicity by NK cells, the down-regulation of signaling through the BCR, and the release of TNF-α and other mediators by macrophages and mast cells. Our observation that ligation of FcR results in the activation of both endogenous MKK4 and MKK7 in MC/9 mast cells (Fig. 5D) supports previous findings that signaling through the FcR activates JNK in bone marrow-derived macrophages (17). Recent studies have shown that signaling through the JNK pathway is required for the production of TNF-α in MC/9 mast cells (43). As signaling through the FcR activates JNK through MKK4 and MKK7, it will be important to determine their individual roles in the production of TNF-α in mast cells.

CD40 is a member of the TNF receptor family, and ligation of CD40 has been reported to activate JNK in B-cells (15, 16). Signaling through CD40 is critical for the growth, differentiation, and class switching of B lymphocytes (44), and the development of the Th1 subset of T lymphocytes through the activation of macrophages and dendritic cells (45). Our findings that ligation of CD40 activated both endogenous MKK4 and MKK7 (Fig. 5D) is consistent with our findings on the activation of endogenous MKK4 and MKK7 in HeLa cells treated with TNF-α (Fig. 5B). MKK7 has also been reported to be activated by ligation of Fas, another member of the TNF receptor family (46). Together these findings suggest that activation of MKK7 may be a common feature of signals activated by ligation of members of the TNF receptor family. Recent reports have shown that the genetic disruption of mkk4 in B lymphocytes failed to affect their growth or their ability to undergo class switching (47). It will be interesting to determine the role of MKK7 in the physiological regulation of the immune system via signaling through the BCR, the FeR, or CD40.

The existence of multiple activators of JNK responsive to different stimuli was established by the fact that MKK4-deficient ES cells still exhibited activation of JNK in response to hyperosmolarity and UV light but not heat shock or anisomycin (19–20). In that MKK7 is activated by all of the above stimuli (Figs. 4 and 5), and MKK7 mRNA is present in ES cells (Table I), the failure of MKK4-deficient ES cells to activate JNK in response to heat shock or anisomycin is paradoxical. It is possible that, although MKK7 is expressed in ES cells, it is not activated by heat or anisomycin because of cell-specific differences in upstream activators. This notion is supported by a recent report that in KB and U937 cells TNF-α activates MKK7 but not MKK4 (34, 37), whereas in HeLa cells we observed that TNF-α activates both MKK7 and MKK4 (5B), an observation recently reported by Wu et al. (36). MKK4 has also been recently reported to be activated in normal bone marrow-derived macrophages treated with TNF-α, supporting the notion that these kinases will be regulated differently depending on their cellular context (48). Furthermore, Wu et al. (36) have reported that ASK1 and GCK activate MKK7 in preference to MKK4, whereas MEKK1 and MEKK2 activate both MKK4 and MKK7 to comparable levels.

The functional significance of the activation of MKK7 is unclear, but its activation by physiological stimuli such as IL-3 (Figs. 4C and 5C), by ligation of immunoregulators such as CD40 (Fig. 5D), BCR (Fig. 5D), FcR (Fig. 5E), CD3 (36), or by the GTPases RAS, RAC, and CDC42 (Fig. 6) suggest that the role of MKK7 will not be confined to stress responses. The existence of an ortholog of MKK7 in C. elegans and our failure to identify an ortholog in S. cerevisiae suggest that MKK7 arose in evolution during the transition from single cellular to multicellular organisms. The notion that MKK7 is involved in processes important for multicellular organisms such as embryonic development, chemotaxis, or apoptosis is in keeping with evidence that mutations in dHep and Bsk, the Drosophila homolog of JNK1, result in a similar failure of epithelial cell movement and dorsal closure (26, 49, 50). Holland et al. (33) have demonstrated that MKK7 is able to partially complement a deficiency of dHep in Drosophila. This demonstrates that MKK7 is highly conserved functionally and suggests that it may play a role in embryological development in mammals. The embryonic lethality resulting from disruption of the mkk4 gene in mice also points to the importance of normal JNK signaling during embryonic development (24–25) and indicates that MKK4 and MKK7 have discrete physiological functions. Future work detailing the function of individual activators of JNK is likely to reveal roles in multiple aspects of development and other physiological processes, including hematopoiesis and immune responses.

Acknowledgments—We thank I. Clark-Lewis for synthesizing cytokines and the peptides used for antibody production; H. Zilten for the rabbit anti-rat immunoglobulin antibody (R108); M. Howard for IC10; P. Young for Flag-tagged CSBP2; L. Zon for the pEPG expression plasmids and the peptides used for antibody production; H. Ziltener for the constructs of JNK1 and SEK1; R. Kay for RASVal-12; R. Cerione for CDC42Val-12; and F. McCormick for RACVal-12. We thank K. Leslie for help with experiments and with the preparation of this manuscript. We thank P. Newburger, J. Anver, and C. Rider for critical reading of the manuscript. This work was supported by a grant from the National Cancer Institute to M. I. Y. We thank the Bikle Family Foundation for Cancer Research and the Canadian Cancer Society for financial support. We thank our colleagues in the laboratory for helpful discussions and for excellent technical assistance.

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