The Stress Kinase Mitogen-activated Protein Kinase Kinase (MKK)7 Is a Negative Regulator of Antigen Receptor and Growth Factor Receptor–induced Proliferation in Hematopoietic Cells

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Abstract

The dual specificity kinases mitogen-activated protein kinase (MAPK) kinase (MKK)7 and MKK4 are the only molecules known to directly activate the stress kinases stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs) in response to environmental or mitogenic stimuli. To examine the physiological role of MKK7 in hematopoietic cells, we used a gene targeting strategy to mutate MKK7 in murine T and B cells and non-lymphoid mast cells. Loss of MKK7 in thymocytes and mature B cells results in hyperproliferation in response to growth factor and antigen receptor stimulation and increased thymic cellularity. Mutation of mkk7 in mast cells resulted in hyperproliferation in response to the cytokines interleukin (IL)-3 and stem cell factor (SCF). SAPK/JNK activation was completely abolished in the absence of MKK7, even though expression of MKK4 was strongly upregulated in mkk7−/− mast cell lines, and phosphorylation of MKK4 occurred normally in response to multiple stress stimuli. Loss of MKK7 did not affect activation of extracellular signal–regulated kinase (ERK)1/2 or p38 MAPK. mkk7−/− mast cells display reduced expression of JunB and the cell cycle inhibitor p16INK4a and upregulation of cyclinD1. Reexpression of p16INK4a in mkk7−/− mast cells abrogates the hyperproliferative response. Apoptotic responses to a variety of stimuli were not affected. Thus, MKK7 is an essential and specific regulator of stress-induced SAPK/JNK activation in mast cells and MKK7 negatively regulates growth factor and antigen receptor–driven proliferation in hematopoietic cells. These results indicate that the MKK7-regulated stress signaling pathway can function as negative regulator of cell growth in multiple hematopoietic lineages.

Key words: MKK7 • SAPK/JNK • proliferation • stress response • hematopoietic cells

Introduction

During the development of all multicellular organisms, cell fate decisions determine whether cells undergo proliferation and differentiation, or apoptosis. Developmental programs and environmental agents trigger distinct and evolutionarily conserved signal transduction cascades that relay signals mediating proliferation, survival, or death. The mi-
togenous protein kinases (MAPKs)* are a family of
serine/threonine kinases which transduce signals from the
cell membrane to the nucleus in response to a wide range
of stimuli (1, 2). These molecules participate in several
different intracellular signaling pathways that control a spec-
trum of cellular processes, including cell growth, differenti-
ation, transformation, apoptosis, and stress responses (3, 4).
Important members of the MAPK family are the extracellu-
lar signal-regulated kinases ERK1 and ERK2, ERK5, p38 MAPK, and the stress-activated protein kinases
(SAPKs, also known as the c-Jun N-terminal kinases
[JNKs]) (5, 6).

SAPKs/JNKs are activated in response to a variety of
cellular stresses such as changes in osmolarity or metabo-
lism, DNA damage, heat shock, ischemia, shear stress, in-
flammatory cytokines such as TNF and IL-1, and ceramide
(3, 4, 7). Once activated, SAPKs/JNKs regulate gene tran-
scription via phosphorylation of transcription factors, in-
cluding c-Jun, JunD, activating transcription factor (ATF)-2,
nuclear factor of activated T cells (NFAT)4, or ELK-1 (8).
Biochemical analyses have indicated that SAPK/JNKs and
SAPK/JNK-regulated signaling pathways are involved in
cell fate decisions resulting in apoptosis, oncogenic trans-
formation, activation of T and B lymphocytes, induction of
proinflammatory cytokines, regulation of proliferation and
cell cycle arrest, and cardiovascular, renal, and hepatic stress
responses (3, 4). Genetic analyses have confirmed that
SAPKs/JNKs regulate T cell activation (9), Th1/Th2 differ-
entiation (10), neuronal cell death (11), UV-induced cell
death in fibroblasts (12), and dorsal closure during Dro-
sophila development (13).

The prototypical MAPK phosphorylation cascade con-
ists of a MAPK kinase kinase (MAPKKK or MEKK), a
MAPK kinase (MKK), and a MAPK (14, 15). MAPKKK
phosphorylate and activate MKK, dual-specificity protein
kinases that in turn phosphorylate MAPK. Activation of
MAPKs requires phosphorylation of both the threonine
and tyrosine residues in the Thr-X-Tyr motif. Several
proteins are a family of

Materials and Methods

mkk7 Gene Targeting and Construction of Somatic Chimeras. A
12-kb genomic mkk7 fragment was isolated from a 129/Ola
mouse library and inserted into the NotI site of pBlueScript II. A
targeting vector was constructed containing a 709-bp short arm
and a 5.4-kb long arm of homology flanking a neomycin resis-
tance cassette (Neo) inserted into the EcoRI/Bgl-II sites of the
genomic clone in antisense orientation to mkk7 transcription.
The linearized construct was electroporated into 107 E14K ES
cells derived from 129/Ola mice. ES cell colonies resistant to
G418 [0.3 mg/ml] were screened for homologous recombina-
tion and proliferation of other hematopoietic cells is not
known. We generated mkk7−/− chimeric mice using re-
combination activating gene (rag)1 blastocyst complementa-
tion and a gene targeting strategy that allowed us to ana-
lyze the function of MKK7 in T and B lymphocytes and non-lymphoid bone marrow mast cell lines (BMMCs).
mkk7−/−/rag1−/− chimeric mice have enlarged thymi, and
thymocytes from these mice hyperproliferate in response to
antigen receptor stimulation. Similarly, mkk7 deficiency
leads to hyperproliferation of B cells and BMMCs after
stimulation of antigen or growth factor receptors. Mutation
of mkk7 in BMMCs results in loss of expression of JunB
and the cell cycle inhibitor p16INK4a and upregulation of
cyclinD1. In BMMCs, MKK7 is required to relay signaling in
response to IgE, anisomycin, UV-irradiation, or NaCl
stimulation to activation of SAPKs/JNKs. Ectopic reex-
pression of p16INK4a in mkk7−/−/ mast cells abrogates the
hyperproliferative response. This study shows that a stress
signaling kinase, i.e., MKK7, can negatively regulate the
proliferation of multiple hematopoietic cell lineages.

Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D;
BMMC, bone marrow mast cell; DN, double negative; DP, double posi-
tive; ERK, extracellular signal-regulated kinase; ES, embryonic stem;
JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase;
MKK, MAPK kinase; rag, recombination activating gene; SAPK, stress-
activated protein kinase; SCF, stem cell factor; SP, single positive.
sham Pharmacia Biotech), and hybridized with probes specific for mkk7 (22). All data presented in this study were obtained from two independently derived mkk7−/− ES cell clones, and all results were comparable between them. If not otherwise stated, all mice used for experiments were between 6–10 wk of age. All mice were maintained at the animal facilities of the Ontario Cancer Institute under specific pathogen-free conditions according to institutional guidelines.

**Immunocytochemistry.** Single cell suspensions of thymi, lymph nodes, bone marrow, blood, and spleens from mkk7−/−, mkk7+/+ or mkk7+/−/rag l−/−, and mkk7−/−/rag l−/− chimeras were stained with FITC-, PE-, or biotin–conjugated Abs reactive to: Thy1.2, CD3ε, CD4, CD8, CD25, CTLA4, CD28, CD95 (FAS), CD5, CD44, CD45, CD69, CD11a (LFA-1), CD11b (Mac-1), MHC class II (I-Ab), CD23, B220, CD43, slgM, slgD, CD19, or Gr-1 (all Abs were from BD Pharmingen). For the analysis of thymocyte precursors, single cell suspensions were stained with PE-conjugated anti-CD4, anti-CD8, anti-CD3ε, anti-B220, anti-CD11b, anti-Gr-1, and anti-TCRβ. FITC-conjugated anti-CD25; and biotin–conjugated anti-CD44. PE-negative precursor cells ( triple-negative thymocytes) were analyzed for expression of CD25 and CD44. Biotinylated Abs were visualized using streptavidin-RED670 (GIBCO BRL). All samples were analyzed by flow cytometry using a FACSscan (Becton Dickinson).

**Lymphocyte Proliferation, Cytokine Production, and Thymocyte Apoptosis.** For proliferation assays, purified lymph node T cells (10⁶ cells/well) were placed into round bottom 96-well plates (Nunc) in freshly prepared IMDM (10% FCS, 10 μM β-mercaptoethanol) and stimulated with PMA plus the Ca²⁺ ionophore A23677, soluble anti-CD3ε (clone 145-2C11, hamster IgG), soluble anti-CD28 (clone 37.51, BD Pharmingen), or the mitogen Con A (Amer sham Pharmacia Biotech). B cells (10⁵ cells/well) were stimulated with anti-IgM (61-6800; Zymed Laboratories), anti-IgM F(ab')2 fragment (61-5900; Zymed Laboratories), or anti-CD40 (clone 145-2C11; BD Pharmingen), or LPS (Sigma-Aldrich). Cells were stimulated in triplicate for different time periods and pulsed for the last 12 h with 1 μCi/well [³H]thymidine (Amer sham Pharmacia Biotech). [³H]Thymidine incorporation was measured using a β-scintillation counter (Coulter). To determine cytokine production in these cultures, the culture supernatants were removed and assayed in triplicate (Coulter). To determine apoptosis, single cell suspensions were stained with PE-conjugated anti-CD4, anti-CD8, anti-CD3ε, anti-B220, anti-CD11b, anti-Gr-1, and anti-TCRβ. FITC-conjugated anti-CD25; and biotin–conjugated anti-CD44. PE-negative precursor cells ( triple-negative thymocytes) were analyzed for expression of CD25 and CD44. Biotinylated Abs were visualized using streptavidin-RED670 (GIBCO BRL). All samples were analyzed by flow cytometry using a FACSscan (Becton Dickinson).

**Signal Transduction.** BMMCs (2 × 10⁶/100 μl) were cultured in medium alone or stimulated with IL-3 (30 ng/ml), NaCl (0.5 M), anisomycin (10 μg/ml), UV-irradiation (500 μJ, using a DNA-Stratalinker; Stratagene), heat shock (45°C in a prewarmed waterbath), cycloheximide (50 μg/ml), or TNFα (100 ng/ml), for various time periods. For FcεR stimulation, cells were incubated with anti-DNP IgE mAb (7 μg/ml, clone SPE7; Sigma-Aldrich) for 1 h on ice, followed by the addition of 50 ng/ml DNP (Sigma-Aldrich) at 37°C to crosslink the FcεR. Cells were pelleted by centrifugation and resuspended in 100 μl ice cold lysis buffer (1% Triton X-100, 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 30 mM Na₂HPO₄, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na₃VO₃). Cell lysates were fractionated by SDS-PAGE and the proteins transferred to PVDF membranes and immunoblotted. Activation of p38 MAPK, ERK1/2, and PKB/Akt was detected using Abs specific for phospho-p38 MAPK (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), phospho-PKB/Akt (Ser473 and Thr308), or phospho-mkk7 (Thr223; Sigma-Aldrich) for 1 h on ice, followed by the addition of 50 ng/ml DNP (Sigma-Aldrich) at 37°C to crosslink the FcεR. Cells were pelleted by centrifugation and resuspended in 100 μl ice cold lysis buffer (1% Triton X-100, 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 30 mM Na₂HPO₄, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na₃VO₃). Cell lysates were fractionated by SDS-PAGE and the proteins transferred to PVDF membranes and immunoblotted. Activation of p38 MAPK, ERK1/2, and PKB/Akt was detected using Abs specific for phospho-p38 MAPK (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), phospho-PKB/Akt (Ser473 and Thr308), or phospho-mkk7 (Thr223; New England Biolabs, Inc. or Upstate Biotechnology), because phosphorylation at these sites has been shown to induce activation of these kinases. To verify equivalent loading and to confirm the identity of the phosphorylated proteins, membranes were stripped and blotted with Abs to non-phosphorylated PKB/Akt, ERK1/2, SAPKs/JNks, p38 MAPK (all from Santa Cruz Biotechnology, Inc.), and MKK4 (Upstate Biotechnology) or actin (Sigma-Aldrich). Protein levels of p16INK4a, p27, actin, JunB, cyclinD1 (Santa Cruz Biotechnology, Inc.), and MKK7 (BD Transduction) were determined in total cell lysates by immunoblotting followed by visualization using enhanced chemoluminescence (ECL). For detection of SAPK/JNK kinase activity, total SAPKs/JNks were immunoprecipitated (1 h, 4°C) from BMMC lysates using polyclonal rabbit anti-SAPK/JNK IgG reactive against all SAPK/JNK isoforms (5). Immune complexes were harvested on protein A-Sepharose beads. For kinase assays, immune complexes were washed three times with the lysis buffer. The beads were resuspended in 20 μl kinase buffer (10 mM MgCl₂, 50 mM Tris-Cl, pH 7.5, 1 mM EGTA, pH 7.5) and SAPK/JNK activity was assayed at 30°C for 30 min in the presence of 1 μCi [γ-³²P]ATP using 5 μg GST-
c-Jun as the in vitro substrate. The reaction was stopped by the addition of 5× SDS sample buffer. GST-c-Jun phosphorylation was visualized by autoradiography as described previously (5).

Results

Targeted Disruption of the Mouse mkk7 Gene. To disrupt the murine mkk7 gene in E14K ES cells, a portion of exon 9, including the phosphorylation motif, was replaced with a PGK-Neo cassette (Fig. 1 A). Because homozygous inactivation of the mkk7 gene in mice leads to embryonic lethality (reference 10, and our own unpublished data), we targeted the second allele in mkk7+/− PGK-Neo ES cells using a different targeting vector containing a hygromycin resistance cassette (Hygro; Fig. 1 B). This vector targeted a different region (parts of exons 13 and 14) of the mkk7 gene, allowing us to ascertain homologous recombination at both alleles (Fig. 1 C). Disruption of both mkk7 alleles in ES cells was confirmed by Southern blot analysis of genomic DNA (Fig. 1 D) and Western blotting (Fig. 1 F).

To study the development and activation of T and B cells in the absence of MKK7, two different mkk7−/− (mkk7−/−neo/hyg) ES cell lines were used to generate mkk7−/−/rag1−/− chimeric mice via rag1 blastocyst complementation (23). The capacity for hygromycin selection also permitted us to generate mkk7−/− BMMCs from the chimeric mice. Loss of mkk7 mRNA expression in several different cell types from mkk7−/−/rag1−/− mice was confirmed by Northern blot analysis. No detectable mkk7 mRNA expression could be found in RNA isolated from mutant ES cells (not shown), thymocytes, lymph nodes, or two different BMCC lines (Fig. 1 E). Absence of mkk7 mRNA expression was confirmed by reverse transcription (RT)-PCR (not shown).

Thus, our gene targeting strategy provided us with a means of analyzing the role of MKK7 in multiple hematopoietic cell lineages, i.e., T- and B-lymphocytes and BMCCs.

Increased Thymocyte Proliferation and Thymic Cellularity in mkk7−/−/rag1−/− Mice. Although mkk7−/−/rag1−/− chimeric mice showed normal numbers and subpopulations of peripheral T cells, we were surprised to observe that these animals displayed thyMICs of significantly increased cellularity (Table I). The proportions of CD4−CD8− double negative (DN) progenitor cells, immature CD4+CD8− double positive (DP) cells, and mature CD4+ and CD8+ single positive (SP) thymocytes were equal in mkk7−/−/rag1−/− and mkk7−/−/rag1−/− thyMICs (Fig. 2A). Moreover, there were no differences in surface expression levels of TCRα/β, CD3, CD4, CD8, CD44, CD28, CD45, TRCVβ subclasses, or CD95 on SP or DP thymocytes (not shown). The maturation of DN precursor populations as defined by c-Kit, CD44, and CD25 surface expression, and the maturation of immature DP thymocytes to mature SP thymocytes as defined by CD69, CD44, HSA, CD5, and H2-Kb expression, were also similar in mkk7−/−/rag1−/− and mkk7−/−/rag1−/− thymocyte populations. Thus, MKK7 is not required for thymocyte development or thymic positive selection. However, loss of MKK7 leads to increased thymic cellularity.

Thymic cellularity is maintained by a balance between apoptosis and cellular proliferation, activities that in many cell types involve SAPK/JNK signaling. To examine the impact of MKK7 deficiency on thymocyte survival, we evaluated the responses in vitro of mkk7−/−/rag1−/− thymocytes to treatment with anti-CD3ε or anti-CD95, stimuli known to induce the apoptotic death of DP thymocytes. No apparent differences in the kinetics or extents of cell death were observed between mutant and control thymocytes after stimulation of either CD3ε (Fig. 2 B) or FAS (Fig. 2 C). Furthermore, the susceptibility of mkk7−/− thymocytes to apoptosis induced by anisomycin, the PI3K inhibitor LY294002, sorbitol (osmotic shock), or dexamethasone was comparable to that of mkk7−/+ chimeric thymocytes.
mocytes (Fig. 2 D). However, mkk7-deficient thymocytes exhibited hyperproliferation in response to antigen receptor engagement (Fig. 2 E). These data imply that antigen receptor–triggered hyperproliferation of thymocytes, and not reduced cell death, accounts for the increased thymic cellularity in mkk7<sup>−/−</sup>→rag1<sup>−/−</sup> chimeric mice. However, as mainly single positive thymocyte proliferate in our assays, we cannot exclude the possibility that MKK7 controls thymocyte cell death in response to a yet unidentified signals that controls cellularity. Moreover, MKK7 could control proliferation of DN progenitor cells.

**Activation of mkk7-deficient Peripheral T Cells.** The SAPK/JNK signaling pathway has been shown to integrate antigen receptor and costimulatory signals in primary T cells and T cell lines (30) and to control Th1/Th2 differentiation (10, 31). It has also been reported that T cells bearing mutations of either mkk4 or jnk2 display partial defects in cell proliferation and IL-2 production in response to suboptimal stimulation of the TCR plus CD28 (9, 32). In addition, transfection and pharmacological inhibitor studies have suggested that MKK7 might regulate production of IL-2 in T cell lines (33). On the other hand, it has been reported in mkk7<sup>−/−</sup> and jnk1/jnk2 double mutant T cells that the MKK7–SAPK/JNK signaling pathway has no role in T cell activation but rather influences effector functions (10). Moreover, the same group reported that mkk7 and jnk1/jnk2 double mutant T cells hyperproliferate and produce more IL-2 in vitro stimulation assays (10). We therefore explored whether the absence of MKK7 had a similar effect on the numbers, activation, or function of peripheral T cells in our chimeric mice. Lymph nodes and spleens of

<table>
<thead>
<tr>
<th>Subsets</th>
<th>mkk7&lt;sup&gt;+/−&lt;/sup&gt;→rag1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>mkk7&lt;sup&gt;−/−&lt;/sup&gt;→rag1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Thymus</td>
<td></td>
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<tr>
<td>Total cell number (×10⁷)</td>
<td>9.84 ± 2.2</td>
<td>22.5 ± 4.6</td>
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<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>81.2 ± 7.6</td>
<td>88.9 ± 2.3</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt; (%)</td>
<td>11.8 ± 5.0</td>
<td>7.60 ± 1.2</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>2.5 ± 0.8</td>
<td>1.2 ± 0.4</td>
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| Spleen  |                               |                               |
| Total cell number (×10⁷) | 2.90 ± 0.6                    | 2.74 ± 0.4                    |
| TCRα/β<sup>+</sup> (%) | 36.3 ± 4.3                    | 33.8 ± 4.9                    |
| B220<sup>+</sup>dgM<sup>+</sup> (%) | 29.9 ± 5.4                    | 27.8 ± 5.6                    |

| Lymph node |                               |                               |
| Total cell number (×10⁷) | 2.96 ± 0.4                    | 3.14 ± 0.3                    |
| CD4<sup>+</sup>CD8<sup>−</sup> (%) | 53.2 ± 4.0                    | 43.6 ± 2.6                    |
| CD4<sup>+</sup>CD8<sup>+</sup> (%) | 19.7 ± 1.6                    | 15.5 ± 2.8                    |

7–12-wk-old mkk7<sup>+/−</sup>→rag1<sup>−/−</sup> and mkk7<sup>−/−</sup>→rag1<sup>−/−</sup> mice were used. Total cells from thymi (n = 8), spleen (n = 5), and lymph nodes (n = 5) were stained with Abs against the indicated proteins and populations determined by FACScan<sup>™</sup>. Bold numbers indicate statistically significant differences between mkk7<sup>+/−</sup> and mkk7<sup>−/−</sup> chimeric mice (Student’s t test; P < 0.05). Values are given as the mean ± S.E.M.

**Figure 2.** Loss of MKK7 expression results in increased thymic cellularity and thymocyte hyperproliferation. (A) Immumocytometric analysis of thymocytes in mkk7<sup>+/−</sup> and mkk7<sup>−/−</sup> chimeric mice. Cells were isolated from 6-wk-old mice and stained with anti-CD4 and anti-CD8. Percentages of positive cells within a quadrant are indicated. One result representative of eight independent experiments is shown. (B–D) Induction of cell death in thymocytes from mkk7<sup>+/−</sup> and mkk7<sup>−/−</sup> chimeric mouse. Freshly isolated thymocytes were stimulated for 20 h with (B) the indicated concentrations of anti-CD3<sub>ε</sub>; (C) anti-CD95 (anti-FAS); and (D) anti-CD95 (anti-FAS) and (D) anti-CD95 (anti-FAS) and (D) anti-CD95 (anti-FAS) and (D) anti-CD95 (anti-FAS). Thymocytes were stained with anti-CD4–PE, anti-CD8–FITC, and the vital dye 7-AAD, and viable CD4<sup>+</sup>CD8<sup>−</sup> cells (7-AAD−) were determined by FACScan<sup>™</sup> in triplicate. Values represent mean percentages of viable CD4<sup>+</sup>CD8<sup>−</sup> DP thymocytes and are normalized to the percentage of viable DP cells in untreated cultures (100%). Spontaneous apoptosis was comparable between mkk7<sup>+/−</sup> and mkk7<sup>−/−</sup> thymocytes both at the start of culture and after 20 h (not shown). One result representative of eight independent experiments is shown for each activation. (E) Thymocyte proliferation. Thymocytes (10⁵/well) were isolated from mkk7<sup>+/−</sup> and mkk7<sup>−/−</sup> chimeric mice and activated with the indicated concentrations of plate-bound anti-CD3<sub>ε</sub> mAbs. Proliferation was determined in triplicate (± SD) at 48 h after a 12-h pulse with [3H]thymidine. One result representative of five independent experiments is shown.
were comparable between mkk7+/−/rag1−/− and mkk7+/−/rag1−/−/lymph node T cells. Similarly, IL-2 and IFN-γ production by lymph node T cells was also normal in the absence of MKK7 (not shown). It should be noted that in some experiments we observed increased proliferation as well as increased IL-2 and IFN-γ production by mkk7+/− T cells compared with mkk7+/+ T cells; however, this increased proliferation was never statistically significant. These results show that loss of MKK7 has no apparent effect on peripheral T cell homeostasis, activation or function.

MKK7 Is a Negative Regulator of B Cell Proliferation. SAPK/JNK activation has been implicated in signal transcription downstream of B cell stimulation via CD40 engagement or LPS treatment (34). However, no proliferative or functional defects were apparent in B cells from mice deficient for mkk4, jnk1, or jnk2 (31, 35, 36). mkk7+/−/rag1−/− chimeric mice displayed normal numbers and differentiation of B220+CD25+, B220+CD25−, B220+CD43+, B220+CD43−, B220+slgM+sIgD+, and CD19+slgM+slgD+ B cells in the bone marrow, and normal populations of B220+slgM+sIgD B cells in peripheral lymphoid organs (Table I, Fig. 4 A, and not shown). Basal serum levels of the Ig subclasses IgM, IgG1, IgG2a, IgG3, and IgA were also comparable in mkk7+/−/rag1−/− and mkk7+/+/rag1−/− chimeric mice (not shown). Thus, B cell development in the bone marrow and mature B cell populations in the periphery appear normal in the absence of MKK7.

To determine whether MKK7 is required for B cell activation, we measured B cell proliferation in response to LPS treatment, anti-CD40 Ab, IgM (Fab′)2 crosslinking, and the engagement of IgM plus CD40. Intriguingly, the proliferation of mkk7−/−/rag1−/− B cells was significantly enhanced in response to anti-IgM (Fab′)2 crosslinking (Fig. 4 B). The increased proliferative response was still observed when mkk7−/−/rag1−/− cells were stimulated with the intact anti-IgM Ab, suggesting that the hyperproliferation is not due to impaired negative signals mediated via FcγR1B (not shown). The proliferation of mkk7−/− cells was also markedly enhanced by treatment with LPS, anti-CD40, or anti-IgM (Fab′)2 plus anti-CD40 (Fig. 4 B). Thus, as was true for thymocytes, loss of MKK7 in B cells results in hyperproliferation in response to growth stimuli or engagement of the antigen receptor.

MKK7 Negatively Regulates Growth Factor–dependent Proliferation of Mast Cells. SAPKs/JNKs and their activators MKK4 and MKK7 are expressed in all hematopoietic lineages. However, the role of MKK7 and SAPKs/JNKs in the function of non-lymphoid hematopoietic cells has not been explored using mutational analysis. Our strategy of mutating one mkk7 allele with a Neo-cassette and the other allele with a hygromycin-resistance vector allowed us to study the role of MKK7 in a non-lymphoid hematopoietic lineage, BMMC lines. Because mast cells derived from mkk7−/− ES cells in the chimeric mice (but not those derived from rag1−/− ES cells) were resistant to hygromycin, several BMMC lines known to be mkk7−/− could readily be established (Fig. 1 E). As controls, we generated mkk7+/+ BMMCs from 129/Ola mice, which have
the same genetic background as the mkk7−/− E14 ES cells. BMHCs of both genotypes showed similar expression levels of c-Kit (SCF-R) and the IgE receptor (not shown), two markers characteristic of mature BMHCs (37). Thus, loss of MKK7 expression does not prevent the emergence and differentiation of BMHCs. However, the proliferation of mkk7−/− BMHCs in response to the mast cell growth factor IL-3 (Fig. 5 A) or SCF (c-Kit-ligand; Fig. 5 B) was strikingly increased compared with that of wild-type BMHCs, paralleling the hyperproliferation observed in mkk7-deficient thymocytes and B cells. Again, no significant differences between mkk7+/+ and mkk7−/− BMHCs were observed in apoptosis induced by growth factor deprivation or osmotic stress (NaCl) (not shown), or by anisomycin, heat shock, or UV-irradiation (Fig. 5 C). Similar results were obtained using in vitro differentiated FcεR+ c-Kit+ mast cell lines from mkk7+/+, mkk7−/−, and mkk7−/− ES cells that were selected and differentiated under identical culture conditions (not shown) (28). These data indicate that loss of MKK7 results in the hyperproliferation of both lymphoid and non-lymphoid hematopoietic cells in response to multiple growth factor or antigen receptor stimulation.

At the molecular level, immunoblot analyses of proteins in BMHCs revealed that expression levels of p46 and p56 SAPK/JNK isoforms, p38 MAPK, ERK1/ERK2, PKB/Akt, and actin were comparable in BMHCs, leading to a concomitant increase in p46 (Fig. 6 A). However, expression of the cell cycle inhibitory molecules, p16INK4a, which act principally on cyclinD1 and CDK4–6, was completely abrogated in mkk7−/− BMHCs, leading to a concomitant increase in cyclinD1 expression (Fig. 6 B). Interestingly, whereas expression of c-Jun was comparable between mkk7+/+ and mkk7−/− BMHCs, expression of JunB, which has been shown to upregulate p16INK4a expression (38), was mark-

![Figure 4](image-url)

**Figure 4.** B cells hyperproliferate in the absence of MKK7. (A) Immunocytometric analysis of B cell populations in the bone marrow (top panels) and spleen (bottom panels) of mkk7+/+ and mkk7−/− chimeric mice. Cells were isolated from 6-wk-old mice and double stained with anti-B220 and anti-CD43, or anti-B220 and anti-sIgM. Percentages of positive cells within a quadrant are indicated. (B) Activation of splenic B cells. Purified spleen cells (10⁵/well) from mkk7+/+ and mkk7−/− chimeric mice were incubated for 36 h in medium alone (None) or medium containing LPS (2 μg/ml), anti-IgM (Fab′), (5 μg/ml), anti-CD40 (5 μg/ml), or anti-IgM (Fab′), (5 μg/ml) plus anti-CD40 (5 μg/ml). Cells were labeled with [3H]thymidine for the last 12 h of culture. The mean [3H]thymidine uptake (± SD) of triplicate cultures is shown. Similar results were observed when different seeding numbers were used and at earlier and later time points of activation (not shown). One result representative of five independent experiments is shown.

![Figure 5](image-url)

**Figure 5.** MKK7 regulates BMHC proliferation but not apoptosis. (A and B) Proliferation of mkk7+/+ and mkk7−/− BMHCs. mkk7+/+ and mkk7−/− BMHCs were incubated with increasing concentrations of (A) IL-3 or (B) SCF (c-Kit-Ligand). [3H]Thymidine uptake was determined 24 h after cytokine addition. Similar results were obtained for five separate cultures from five mice of each genotype. (C) Induction of apoptosis in mkk7+/+ and mkk7−/− BMHCs. Cells were stimulated for 14 or 42 h with either anisomycin (Aniso; 10 μM), UV-irradiation (500 mJ), or heat shock (45°C for 30 min). Cell viability was determined in triplicate by 7-AAD and PI staining and normalized to the percentage of viable cells in untreated cultures. One result of a triplicate culture (± SD) representative of five independent experiments is shown for each activation. Normal susceptibility to cell death of mkk7−/− BMHCs (P > 0.1) was also observed at various seeding numbers and in response to growth factor deprivation and osmotic shock (not shown).
MKK7 is essential for SAPK/JNK activation in BMMCs. (A) Western blot analysis of expression levels of p54 and p46 SAPK/JNK, p38 MAPK, ERK1, PKB/Akt, JunB, p16INK4a, p27, and cyclinD1 in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. Actin is shown as a loading control. Total cell lysate proteins (30 μg/lane) were separated by SDS-PAGE and incubated with Abs specific for the indicated molecules. (B) Western blot analysis of expression levels of p16INK4a and actin in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. BMMCs were left untreated (None) or activated with anti-DNP IgE (7 μg/ml), plus DNP (50 ng/ml, 15 min), IL-3 (30 ng/ml, 20 min), or anisomycin (Aniso; 10 μg/ml, 20 min), heat shock (heat; 45°C, 30 min), UV-irradiation (500 mJ, 15 min), NaCl (0.5 M, 10 min), cycloheximide (CHX; 50 μg/ml, 20 min), TNFα (100 ng/ml, 20 min), or TNFα (100 ng/ml) plus CHX (50 μg/ml) for 20 min. Total SAPK/JNK was immunoprecipitated and assayed for in vitro kinase activity using GST-c-Jun as the substrate. In C, the levels of immunoprecipitated p46 and p54 SAPK/JNK are shown as a loading control. One representative of four independent experiments is shown. (E) Expression and phosphorylation (Thr223) of MKK4 in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. BMMCs were stimulated with the indicated agents as in B. Proteins were separated by SDS-PAGE and detected using an Ab reactive to total MKK4 or an Ab specific for phospho-MKK4 (Thr223). Thr223 phosphorylation is indicative of activated MKK4. Actin levels are shown as a loading control.

Figure 6. MKK7 is essential for SAPK/JNK activation in BMMCs. (A) Western blot analysis of expression levels of total p54 and p46 SAPK/JNK, p38 MAPK, ERK1, PKB/Akt, JunB, p16INK4a, p27, and cyclinD1 in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. Actin is shown as a loading control. Total cell lysate proteins (30 μg/lane) were separated by SDS-PAGE and incubated with Abs specific for the indicated molecules. (B) Western blot analysis of expression levels of p16INK4a and actin in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. BMMCs were left untreated (None) or activated with anti-DNP IgE (7 μg/ml) plus DNP (50 ng/ml, 15 min), IL-3 (30 ng/ml, 20 min), or anisomycin (Aniso; 10 μg/ml, 20 min), heat shock (heat; 45°C, 30 min), UV-irradiation (500 mJ, 15 min), NaCl (0.5 M, 10 min), cycloheximide (CHX; 50 μg/ml, 20 min), TNFα (100 ng/ml, 20 min), or TNFα (100 ng/ml) plus CHX (50 μg/ml) for 20 min. Total SAPK/JNK was immunoprecipitated and assayed for in vitro kinase activity using GST-c-Jun as the substrate. In C, the levels of immunoprecipitated p46 and p54 SAPK/JNK are shown as a loading control. One representative of four independent experiments is shown. (E) Expression and phosphorylation (Thr223) of MKK4 in mkk7+/+ (+/+ ) and mkk7−/− (−/−) BMMCs. BMMCs were stimulated with the indicated agents as in B. Proteins were separated by SDS-PAGE and detected using an Ab reactive to total MKK4 or an Ab specific for phospho-MKK4 (Thr223). Thr223 phosphorylation is indicative of activated MKK4. Actin levels are shown as a loading control.

MKK7 Is the Critical Mediator of SAPK/JNK Activation in Mast Cells. The previous findings that SAPKs/JNks could be activated by both MKK4-dependent and MKK4-independent signaling cascades (23–25), suggested that cells can sense particular stresses and trigger distinct signaling paths in response. To investigate whether similar MKK7-dependent or -independent pathways might also control SAPK/JNK activation, mkk7+/+ and mkk7−/− BMMCs were either treated with stress stimuli or activated via engagement of membrane receptors, and the activation of components of stress signaling pathways was assessed.

In wild-type BMMCs, SAPKs/JNks are strongly activated in response to Fcε receptor stimulation (Fig. 6 C). Moreover, SAPKs/JNks are strongly activated after stimulation with anisomycin, UV-irradiation, or NaCl (Fig. 6 D). However, IL-3, anisomycin, heat shock, UV-irradiation, NaCl, cycloheximide, or TNFα used at various doses and in a series of activation protocols failed to activate SAPKs/JNks in mkk7−/− BMMCs (Fig. 6, C and D, and not shown). FcεR stimulation or anisomycin treatment led to normal activation (phosphorylation) of p38 MAPK in mkk7−/− BMMCs. Similarly, activation of ERK1/ERK2 and PKB/Akt induced by IgE plus antigen or IL-3 was comparable in mkk7+/+ and mkk7−/− BMMCs (not shown). Thus, MKK7 is not required for the activation of p38 MAPK, ERK1/ERK2, or PKB/Akt in BMMCs. Interestingly, whereas wild-type BMMCs expressed very low levels of MKK4 protein regardless of stress stimulus, MKK4 was strongly upregulated in mkk7−/− BMMCs (Fig. 6 E). Furthermore, the stimuli NaCl, anisomycin, UV-irradiation, or heat shock, which did not activate SAPKs/JNks in the absence of MKK7 (Fig. 6 D), were able to induce strong activation of MKK4, as detected by an Ab specific for the phosphorylated form of MKK4 (Fig. 6 E). Thus, at least in mast cells, MKK7 expression is essential for SAPK/JNK activation, and upregulated levels of activated MKK4 alone are insufficient.

In Vitro Cooperation of Both MKK4 and MKK7 for Complete SAPK/JNK Activation. The finding that MKK4 alone cannot activate SAPKs/JNks in BMMCs may be explained in two ways. Either MKK4 is irrelevant for this function in BMMCs or MKK4 and MKK7 cooperate in vivo to achieve full SAPK/JNK activation. In an attempt to establish a biochemical basis for cooperation, we designed an in vitro kinase assay system in which purified recombinant MKK4, MKK7, and SAPKβ were mixed together followed by assay of SAPK/JNK activity. Addition of either recombinant wild-type MKK4 or wild-type MKK7 to SAPKβ/JNK3 induced only moderate or low, respectively, levels of SAPKβ/JNK3 kinase activity (Fig. 7). Even addition of a constitutively active form of MKK7 (MKK7DE) induced only moderate SAPKβ/JNK3 kinase activity. However, addition of both MKK4 and MKK7DE resulted in a dramatic and synergistic induction of SAPKβ/JNK3 activation, strong evidence of cooperativity between these two pathway intermediates. Thus, MKK7 is an essential activator of SAPKs/
JNKs in mast cells and its function is not redundant with that of MKK4. Rather, at least in vitro, both MKK4 and MKK7 are necessary for complete SAPK/JNK activation.

Ectopic Expression of p16INK4a Reverts Hyperproliferation of mkk7+/− BMMCs. Mutation of mkk7 in BMMCs results in hyperproliferation, loss of expression of JunB and the cell cycle inhibitor p16INK4a, and upregulation of cyclinD1 (Fig. 6 A). We therefore speculated that mutation of mkk7 in BMMCs may result in hyperproliferation because reduced JunB expression and a loss of p16INK4a expression leads to upregulated cyclinD1 and accelerated cell cycle progression. To address whether p16INK4a has a role in hyperproliferation of mkk7+/− BMMCs, we reexpressed p16INK4a in mkk7−/− deficient BMMCs using a retroviral expression vector (Fig. 8, inset). Importantly, reexpression of p16INK4a suppressed hyperproliferation of mkk7−/− BMMCs (Fig. 8). This result suggests that mkk7 deficiency might cause downregulation of p16INK4a and subsequent hyperproliferation of BMMCs.

Discussion

SAPKs/JNKs are activated in response to a variety of cellular and environmental cues. To determine the role of the SAPK/JNK activator MKK7 in hematopoietic cells, we mutated both mkk7 alleles in ES cells using two selectable markers and generated mkk7−/− deficient chimeric mice via rag1 complementation. Surprisingly, mkk7−/−/rag1−/− chimeric mice exhibit markedly enlarged thymi and thymocyte hyperproliferation. mkk7−/− deficient mature B cells and mast cell lines also hyperproliferate in response to cytokine and antigen receptor stimulation, but respond normally to death stimuli. In mast cells, the absence of MKK7 results in enhanced cell cycle progression, increased expression of cyclinD1, and significantly reduced expression of the cyclin-dependent kinase inhibitor p16INK4a. In contrast, a deficiency of MKK7 does not affect the expression of p27 nor the activation of ERK1/2 or p38 MAPK. Reexpression of p16INK4a reduced the hyperproliferative phenotype in mkk7−/− mast cells. Intriguingly, although MKK4, the second direct SAPK/JNK activator, is strongly upregulated in mkk7−/− mast cell lines and activated in response to multiple stimuli, SAPK/JNK activation was still completely abolished in response to these same stimuli in the absence of MKK7. Thus, MKK7 is essential for SAPK/JNK activation in mast cells and MKK7 acts as a negative regulator of growth factor– and antigen receptor–induced proliferation of different hematopoietic cell lineages.

The engagement of growth factor receptors or stimulation by mitogens induces the activation of ERK1 and ERK2 in many cell types and these kinases regulate the activation of transcription factors governing cellular proliferation (39). SAPKs/JNKs are activated in response to a variety of environmental and cellular stresses such as metabolic changes or DNA damage. These stresses can result in cell cycle arrest to allow for repair. However, other than a positive regulatory role for MKK4 and JNK2 in antigen receptor and CD28 costimulation-dependent proliferation of mature T cells (9, 32), there is little in vivo data to suggest that components of the stress signaling pathway can in fact negatively regulate cell growth. Our results show that loss of a stress signaling kinase, MKK7, results in hyperproliferation of thymocytes, mature B cells, mast cells (our results), and possibly T cells (10). This hyperproliferation of mkk7−/− hematopoietic cells appears to be due, not to impaired cell death, but rather to enhanced cell cycle progression.

SAPKs/JNKs can associate with all three members of the Jun-family of transcription factors, c-Jun, JunB, and JunD. These molecules probably have specific and distinct functions in cellular proliferation and depending on the stimulus and cell type, can also mediate differentiation, cell death, and/or growth arrest (40). In fibroblasts, expression
of c-Jun has a positive effect on proliferation (41), whereas Jun-D (42) and JunB (38) negatively regulate growth. It has been recently shown that increased JunB expression in 3T3 fibroblasts induces high levels of p16INK4a but that other cell cycle inhibitors are not affected (38). On the other hand, c-Jun overexpression inhibits p16INK4a transcription. Our data show that mkk7−/− BMMCs display reduced expression of JunB, completely lack expression of p16INK4a, leading to dramatic upregulation of cyclinD1 expression. Thus, in the absence of MKK7, impaired c-Jun and JunD phosphorylation and/or lower expression of and deregulated JunB activity might explain the observed loss of p16INK4a expression and increase in cell growth. The competing regulatory influences of JunB and c-Jun on p16INK4a expression provide a molecular framework within which SAPK/JNKs could conceivably control cell growth (38). Consistent with this hypothesis, reexpression of p16INK4a suppressed hyperproliferation of mkk7−/− mast cells. However, we observed normal p16INK4a expression in in vitro differentiated B cells. Thus, it needs to be determined whether, similar to BMMCs, hyperproliferation of mkk7−/− B cells and thymocytes might be also regulated by p16INK4a. Moreover, our data in mkk7−/− B cells suggest that other molecular targets than p16INK4a exist that control negative regulation of cell cycle progression downstream of MKK7. Identification of such targets should be of interest to the understanding of development and function of hematopoietic lineages as well as the understanding of cellular transformation in leukemias.

It has been proposed that SAPK/JNK activation triggers apoptosis in response to many types of stress, including UV and γ-irradiation, protein synthesis inhibitors, high osmolality, toxins, ischemia/reperfusion injury in heart attacks, heat shock, anti-cancer drugs, ceramide, peroxoide, and inflammatory cytokines (3, 4). Several lines of evidence support this view. The overexpression of dominant negative MKK4 can block the induction of cell death by heat shock, irradiation, anti-cancer drugs, peroxides, ceramide, or cytokine deprivation (43, 44). In addition, overexpression of inactive c-Jun or dominant negative MEKK1 inhibits the induction of apoptosis by irradiation, ceramide, or heat shock in U937 and BAE cells (43), and protects PC12 cells from apoptosis triggered by nerve growth factor (NGF) withdrawal (45). These results suggested that the MKK7−/− SAPK/JNK −/− c-Jun signaling cascade can transduce proapoptotic signals.

However, recent studies of genetic “knockouts” of SAPK/JNK isoforms and MKK4 have demonstrated that MKK4 and SAPK/JNK activation are not essential for the induction of cell death in response to all apoptotic stimuli. For example, SAPK/JNK3 knockout mice are viable but display a specific defect in kainate-induced apoptosis of hippocampal neurons (11). Similarly, double mutation of jnk1/ jnk2 in primary murine fibroblasts protects them against UV- and anisomycin-induced apoptosis (12). In contrast, we (46) and others (25) have previously reported defective liver formation and massive hepatocyte apoptosis in mouse embryos lacking MKK4. In this case, MKK4 provides a crucial and specific survival signal for hepatocytes during embryonic morphogenesis. Additional genetic analyses of mkk4−/− ES cell clones and mouse embryonic fibroblasts have confirmed that both MKK4-dependent and MKK4-independent pathways for SAPK/JNK activation exist (23–25). MKK4 is the critical activator of SAPKs/JNKs in response to anisomycin and heat shock, whereas SAPK/JNK activation in response to osmolarity changes, UV-irradiation, γ-irradiation, or ceramide is independent of MKK4, at least in these cells. Our experiments in mkk7−/− BMMCs show that MKK7 is required for UV-, anisomycin-, and NaCl-induced SAPK/JNK activation. However, the kinetics and extent of UV-, anisomycin-, and NaCl-induced apoptosis were comparable in mkk7−/− and mkk7+/+ BMMCs and thymocytes. Thus, although JNK1/2 might be required to mediate UV- and anisomycin-triggered cell death in fibroblasts (12), MKK7-controlled SAPK/JNK activation does not have any apparent role in the apoptotic response of mast cells and thymocytes to the same stimuli. We conclude that, rather than being essential for apoptosis, the MKK7−/− SAPK/JNK−/− pathway modulates the death response in a stimulus- and cell type-specific manner.

Signaling pathways for SAPK activation may also be developmentally regulated during lymphopoiesis. PMA/ Ca²⁺-ionophore stimulation can induce SAPK/JNK activation in mature T cells from mkk4−/−rag2−/− chimeric mice, but not in immature thymocytes (32). Perhaps not coincidentally, immature thymocytes express high levels of MKK7 and low levels of MKK4, whereas mature T cells express high levels of MKK4 and low levels of MKK7 (47, 48). Cell type-specific variation in expression of MKK7 and MKK4 in thymocytes versus mature T cells could explain the normal activation of mature lymph node T cells but enhanced thymic cellularity and thymocyte hyperproliferation observed in our mkk7−/− rag1−/− chimeric mice.

The finding that different types of stress or different stages of development trigger distinct signaling pathways for SAPK/JNK activation has been explained by differential activation of MKK4 and MKK7 via upstream kinases, and/or differential scaffolding of the MKK4 and MKK7 signaling pathways via adaptor molecules (49). It has further been proposed that cells can sense different types of endogenous or environmental stress signals and that MKK4- and MKK7-mediated pathways of SAPK/JNK activation are controlled by distinct “transducisomes”; that is, they are structurally and/or biochemically separated (49). However, it has also been reported that JNK1 (50) and JNK3 (51) are synergistically activated in vitro by the presence of both MKK4 and MKK7, suggesting that complete activation of SAPK/JNK enzymatic activity may sometimes require phosphorylation by two different MKKs. This situation bears resemblance to recent findings that two separate binding and phosphorylation events by MEK to its substrate ERK may be required for complete ERK activation (52, 53).

The results of our in vitro kinase assays (Fig. 6 D) provide further evidence that MKK4 and MKK7 must cooperate to fully activate SAPKs/JNKs. Functional synergy between MKK4 and MKK7 could explain why overexpression of
dominant inhibitory MKK4 or dominant-negative MKK7 inhibits activation of SAPK/JNK in response to multiple stimuli. Whether the synergy between MKK4 and MKK7 in SAPK/JNK activation is a universal mechanism used by other MKK isoforms needs to be tested. Importantly, our results in mkk7−/− mast cells show that MKK7 expression is required for SAPK/JNK activation in response to all stimuli tested despite the fact that MKK4 expression is upregulated and MKK4 is strongly phosphorylated. Impaired SAPK/JNK activity in mkk7−/− mast cells despite increased phosphorylation of MKK4 could be explained by functional synergy between MKK4 and MKK7 in these cells in vivo. Alternatively, MKK7 itself and/or an MKK7-associated molecule could provide a scaffold for the interaction between MKK4 and SAPK/JNKs. Such a mechanism has been reported for Jun-B and c-Jun in which Jun-B can recruit c-Jun to SAPKs/JNKs (54).

In conclusion, our data provide evidence that the stress signaling kinase MKK7 is a negative regulator of growth factor and antigen receptor–driven proliferation in hematopoietic cells. We have also demonstrated that MKK7 is essential for SAPK/JNK activation in mast cells.

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