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-

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togen-activated 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 spectrum of cellular processes, including cell growth, differentiation, transformation, apoptosis, and stress responses (3, 4). Important members of the MAPK family are the extracellular 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 metabolism, DNA damage, heat shock, ischemia, shear stress, inflammatory cytokines such as TNF and IL-1, and ceramide (3, 4, 7). Once activated, SAPKs/JNKs regulate gene transcription via phosphorylation of transcription factors, including 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 transformation, 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 differentiation (10), neuronal cell death (11), UV-induced cell death in fibroblasts (12), and dorsal closure during *Drosophila* development (13).

The prototypical MAPK phosphorylation cascade consists 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 mammalian MKKs have been identified to date, including MEK1, MEK2, MKK3, MKK4 (also known as SEK1 or JNKK1), MEK5, MKK6 (16–18), and MKK7 (SEK2, JNKK2; references 19–22). SAPKs/JNKs are activated directly by MKK4 and MKK7, but by no other MKKs. Recent studies of murine embryonic stem (ES) cells lacking the *mkk4* gene (23–25) have demonstrated that MKK4 is critical for the activation of SAPKs/JNKs in response to the protein synthesis inhibitor anisomycin or heat shock. However, SAPK/JNK activity was still inducible in *mkk4*-deficient ES cells subjected to UV-irradiation or changes in osmolarity. Loss of MKK7 in ES cells abrogates, but does not abolish, SAPK/JNK activation in response to osmolarity changes, UV-irradiation, heat shock, or anisomycin (10). In addition, D-MKK4 and D-MKK7 serve nonredundant functions in *Drosophila* (3). These results implied that the SAPK/JNK activators MKK7 and MKK4 operate independently and that different stresses utilize distinct MAPK signaling pathways to activate SAPKs/JNKs.

Recently is has been shown that *mkk7*-deficient and *jnk1/jnk2* double mutant T cells hyperproliferate in response to antigen receptor crosslinking (10), suggesting that the MKK7-regulated stress kinase signaling pathway might negatively regulate T cell activation. Whether genetic inactivation of MKK7 expression has also a role in the activation and proliferation of other hematopoietic cells is not known. We generated $mkk7^{-/-}$ chimeric mice using recombination activating gene (*rag*)*1* blastocyst complementation and a gene targeting strategy that allowed us to analyze 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 reexpression 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.

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 resistance 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 10^7 E14K ES cells derived from 129/Ola mice. ES cell colonies resistant to G418 [0.3 mg/ml] were screened for homologous recombination by PCR using primers specific for *mkk7* genomic sequences and Neo. Recombinant colonies were confirmed by Southern blotting of XbaI-digested genomic DNA hybridized to a 530-bp 3′ flanking probe. E14K ES cells heterozygous for the *mkk7* mutation were retargeted using a hygromycin-resistance cassette that replaced parts of exon 13 and exon 14 of the *mkk7* gene. For construction of somatic chimeras, two independent $mkk7^{-/-}$ ES cell clones ($mkk7^{neo/hyg}$) and a $mkk7^{+/-}$ ($mkk7^{+/neo}$) E14K ES cell clone were injected into 3.5 day $rag1^{-/-}$ blastocysts and transferred to pseudopregnant foster mothers as described (26). As E14 ES cells are derived from 129/Ola mice, age-matched 129/Ola mice were used as wild-type controls in some experiments. For Northern blotting, total RNA (20 μg) was isolated from lymph node cells, thymocytes, spleen cells, and mast cell lines, electrophoresed, transferred to a Hybond-N membrane (Amer-

*Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D; BMMC, bone marrow mast cell; DN, double negative; DP, double positive; 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.

Immunocytometry. Single cell suspensions of thymi, lymph nodes, bone marrow, blood, and spleens from *mkk7*$^{+/+}$, *mkk7*$^{+/-}$ *rag1*$^{-/-}$, and *mkk7*$^{-/-}$*rag1*$^{-/-}$ chimeras were stained with FITC-, PE-, or biotin-conjugated Abs reactive to: Thy1.2, CD3ε, TCRα/β, TCRVβ6, TCRVβ8.1/8.2, TCRVβ14, TCRγ/δ, 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, sIgM, sIgD, 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-TCRα/β; 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 FACScan™ (Becton Dickinson).

Lymphocyte Proliferation, Cytokine Production, and Thymocyte Apoptosis. For proliferation assays, purified lymph node T cells (10^5 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 Ca^{2+} ionophore A23617, soluble anti-CD3ε (clone 145-2C11, hamster IgG), soluble anti-CD28 (clone 37.51; BD PharMingen), or the mitogen Con A (Amersham Pharmacia Biotech). B cells (10^5 cells/well) were stimulated with anti-IgM (61-6800; Zymed Laboratories), anti-IgM $F(ab')_2$ fragment (61-5900; Zymed Laboratories), 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 [^{3}H]thymidine (Amersham Pharmacia Biotech). [^{3}H]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 for the production of IL-2 and IFN-γ using ELISA (R&D Systems). For the analysis of thymocyte apoptosis, single cell suspensions were cultured for various times in 24-well flat bottom tissue culture plates (Nunc) at a density of 2×10^6/ml in IMDM (10% FCS, 10 μM β-mercaptoethanol). The following stimuli were administered: anisomycin (10 μg/ml; Sigma-Aldrich); LY294002 (50 μM; Sigma-Aldrich); dexamethasone (10 nM; Sigma-Aldrich); anti-CD95 (FAS) (Jo2; BD PharMingen); sorbitol (0.4 M; Sigma-Aldrich); NaCl (0.3 M); heat shock (43°C, 30 min); or anti-CD3ε (clone 145-2C11, hamster IgG; BD PharMingen). In the latter case, anti-CD3ε was immobilized on the plates with 10 μg/ml rabbit anti–hamster IgG (Jackson ImmunoResearch Laboratories). At various time points after stimulation, thymocytes were harvested, stained with the vital dye 7-amino-actinomycin D (7-AAD), anti-CD4, and anti-CD8, and subjected to flow cytometric analysis. Apoptosis was confirmed using Annexin V staining to visualize membrane changes, propidium iodide (PI) staining and trypan blue exclusion to test for membrane integrity, and DIOC6[3] staining to test for mitochondrial potential.

BMMC Lines. Bone marrow cells were flushed from femurs of *mkk7*$^{-/-}$*rag1*$^{-/-}$ chimeric and control mice. Cells were washed twice with PBS and resuspended at 5×10^5/ml in Opti-MEM medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, antibiotics (penicillin plus streptomycin; GIBCO/BRL), and 2 ng/ml IL3 (Genzyme). BMMCs were isolated by continuous transfer of cells growing in suspension as described (27). Proliferation of BMMCs was assessed by [^{3}H]thymidine incorporation. In brief, cells were deprived of IL-3 for 12 h, cultured in the presence of various concentrations of IL-3 or stem cell factor (SCF) for 24 h, and pulse-labeled with [^{3}H]thymidine for 12 h. For cell death assays, BMMCs were deprived of IL-3 and/or serum, or stimulated with NaCl, UV-irradiation, anisomycin, or heat shock as above. At various time points after stimulation, apoptosis was determined in triplicate cultures by 7-AAD and Annexin V staining. Cell cycle progression was monitored using PI staining. For infection experiments, pBabe-puro or pBabe-puro–expressing p16INK4a (gift of E. Wagner, Institute for Molecular Pathology, Vienna, Austria) were transfected into a phoenix-E packaging cell line. *mkk7*$^{-/-}$ BMMCs were cultured in the culture supernatant of the packaging cells for 1 d and selected with 2.5 μg/ml puromycin for 1 wk. In vitro generation of B220$^+$IgM$^+$ B cells and FcεR$^+$c-Kit$^+$ mast cells from *mkk7*-mutant ES cell lines was as described (28, 29).

Signal Transduction. BMMCs (2×10^6/100 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 mJ, 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 $Na_4P_2O_7$, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). 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-MKK4 (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/ERK2, 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 $MgCl_2$, 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-^{32}P]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).

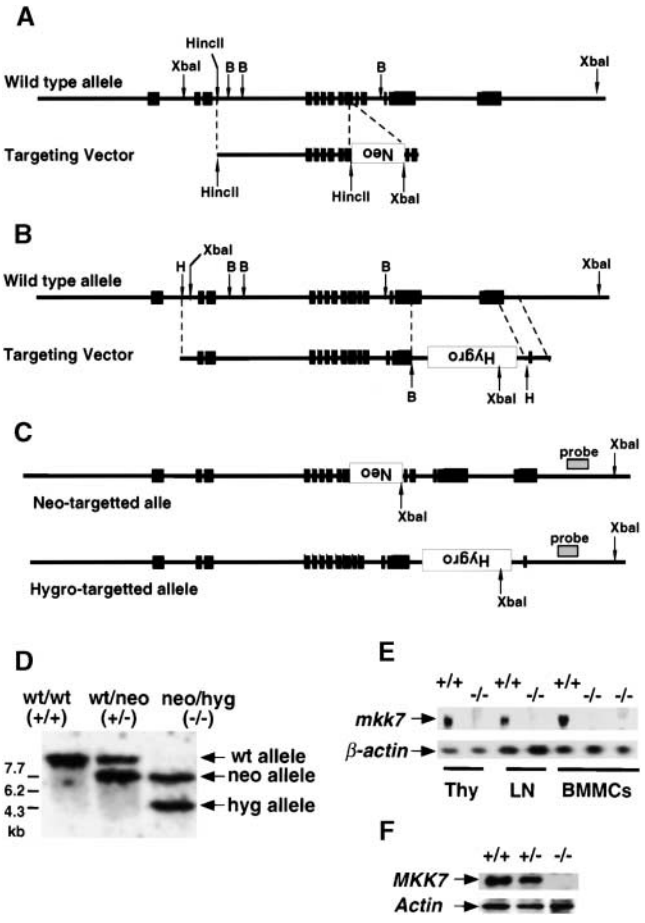

Figure 1. Gene targeting of *mkk7*. (A–C) Partial restriction map of genomic *mkk7* sequences and construction of the neomycin resistance (Neo) (A) and hygromycin resistance (Hyg) (B) insertion vectors. The 14 exons of murine *mkk7* are shown as filled boxes. The genomic *mkk7* flanking probe used for Southern blotting is indicated in C. Restriction enzymes used for construction of the vectors and genomic Southern blotting (XbaI) are indicated. B, BamHI; H, HindIII; E, EcoRI. (D) Genomic analysis of ES cells. Genomic DNA was isolated from wild-type *mkk7*$^{+/+}$ (+/+), *mkk7*$^{+/neo}$ (+/−), and *mkk7*$^{neo/hyg}$ (−/−) E14 ES cells, digested with XbaI, and analyzed by Southern blotting using the 3′ flanking probe indicated in C. Molecular weight markers and the wild-type (wt) and two mutant bands are indicated. (E) Northern blot analysis of *mkk7* mRNA expression in thymocytes (Thy), lymph nodes, and BMMCs isolated from wild-type (+/+) and *mkk7*$^{-/-}$*rag1*$^{-/-}$ (−/−) chimeric mice. Total RNA (20 μg) was probed using full-length *mkk7* or β-actin cDNA. (F) Western blot analysis of MKK7 expression in ES cells.

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 BMMC 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 BMMCs.

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 thymi 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*$^{-/-}$ thymi (Fig. 2 A). Moreover, there were no differences in surface expression levels of TCRα/β, CD3, CD4, CD8, CD28, CD45, TCRVβ 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-K^b 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-FAS (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 thy-

Table I. *Lymphocyte Populations in mkk7$^{-/-}$ Chimeric Mice*

Subsets	*mkk7*$^{+/-}$ →*rag1*$^{-/-}$	*mkk7*$^{-/-}$ →*rag1*$^{-/-}$
Thymus		
Total cell number (×10^7)	9.84 ± 2.2	22.5 ± 4.6
CD4$^+$CD8$^+$ (%)	81.2 ± 7.6	88.9 ± 2.3
CD4$^+$CD8$^+$ (%)	11.8 ± 5.0	7.60 ± 1.2
CD4$^+$CD8$^+$ (%)	2.5 ± 0.8	1.2 ± 0.4
Spleen		
Total cell number (×10^7)	2.90 ± 0.6	2.74 ± 0.4
TCRα/β$^+$ (%)	36.3 ± 4.3	33.8 ± 4.9
B220$^+$sIgM$^+$ (%)	29.9 ± 5.4	27.8 ± 5.6
Lymph node		
Total cell number (×10^7)	2.96 ± 0.4	3.14 ± 0.3
CD4$^+$CD8$^-$ (%)	53.2 ± 4.0	43.6 ± 2.6
CD4$^-$CD8$^+$ (%)	19.7 ± 1.6	15.5 ± 2.8

7–12-wk-old *mkk7*$^{+/-}$→*rag1*$^{-/-}$ and *mkk7*$^{-/-}$→*rag1*$^{-/-}$ 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™. Bold numbers indicate statistically significant differences between *mkk7*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice (Student's t test; $P < 0.05$). Values are given as the mean ± S.E.M.

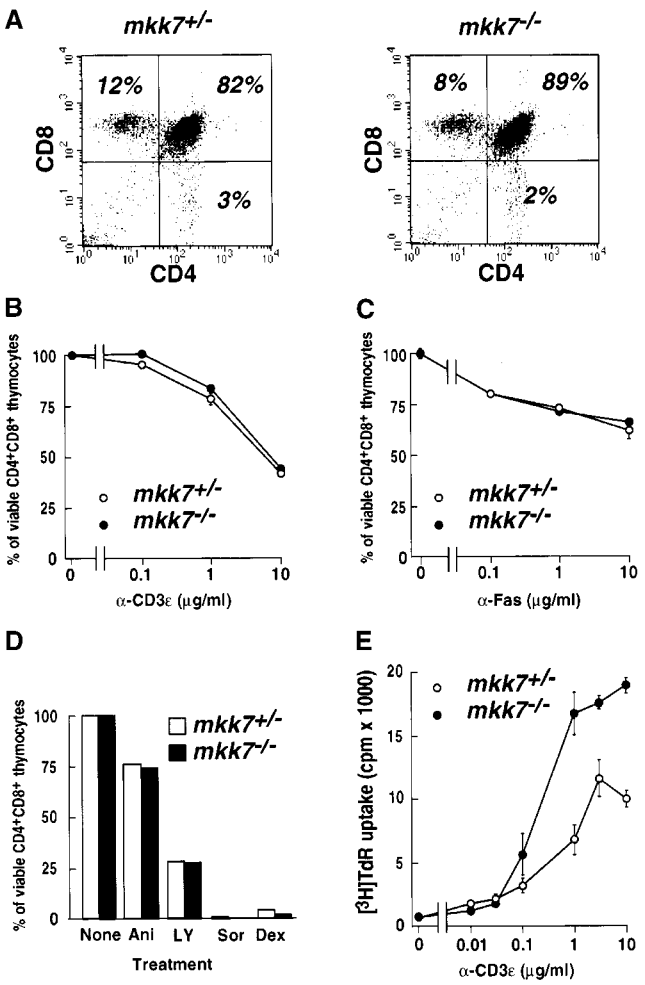

Figure 2. Loss of MKK7 expression results in increased thymic cellularity and thymocyte hyperproliferation. (A) Immunocytometric analysis of thymocytes in *mkk7*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice. Cells were isolated from 6-wk-old mice and stained with anti-CD4 and anti-CD8. Percentages of positive 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*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice. Freshly isolated thymocytes were stimulated for 20 h with (B) the indicated concentrations of anti-CD3ε; (C) anti-CD95 (anti-FAS); and (D) anisomycin (Ani; 10 μg/ml), the PI3K inhibitor LY294002 (LY; 50 μM), sorbitol (Sor; 0.4 M), or dexamethasone (Dex; 10 nM). Thymocytes were stained with anti-CD4–PE, anti-CD8–FITC, and the vital dye 7-AAD, and viable CD4$^+$CD8$^+$ cells (7-AAD–negative) were determined by FACS® in triplicate. Values represent mean percentages of viable CD4$^+$CD8$^+$ DP thymocytes and are normalized to the percentage of viable DP cells in untreated cultures (100%). Spontaneous apoptosis was comparable between *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ 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 (10^5/well) were isolated from *mkk7*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice and activated with the indicated concentrations of plate-bound anti-CD3ε mAbs. Proliferation was determined in triplicate (± SD) at 48 h after a 12-h pulse with [^{3}H]thymidine. One result representative of five independent experiments is shown.

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*$^{-/-}$*rag1*$^{-/-}$ 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, it has been reported in *mkk7* 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 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

mkk7$^{-/-}$*rag1*$^{-/-}$ chimeric mice contained normal numbers and ratios of CD4$^+$ and CD8$^+$ T cells (Table I and Fig. 3 A). Moreover, the surface levels of TCRα/β, CD3, CD4, CD8, CD28, CD45, CD44, LFA1, CD25, and CD69 on both splenic and lymph node CD4$^+$ and CD8$^+$ T cells were comparable in *mkk7*$^{-/-}$*rag1*$^{-/-}$ and *mkk7*$^{+/+}$*rag1*$^{-/-}$ mice (not shown). Thus, despite enhanced thymic cellularity, loss of MKK7 does not affect cell numbers or surface phenotypes of peripheral T cells.

To investigate the role of MKK7 in T cell function, we analyzed the activation and proliferation of peripheral T cells in response to different concentrations of anti-CD3ϵ Ab, anti-CD3ϵ plus anti-CD28 Abs, the mitogen Con A, or PMA/calcium-ionophore, stimuli that bypass the initial antigen receptor signal. At all doses and stimuli tested, the kinetics (not shown) and extents (Fig. 3 B) of proliferation 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.

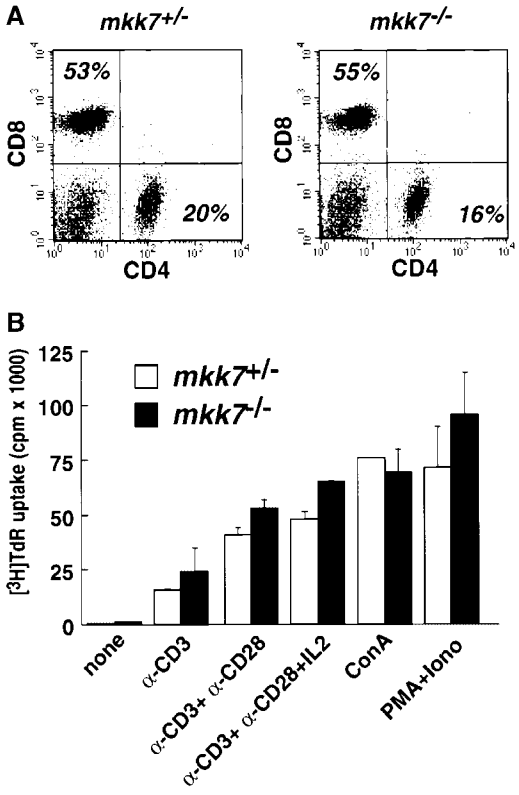

Figure 3. Activation of mature T cells. (A) Immunocytometric analysis of lymph node T cells in *mkk7*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice. Cells were isolated from 6-wk-old mice and stained with anti-CD4 and anti-CD8. Percentages of positive cells within a quadrant are indicated. One result representative of five independent experiments is shown. (B) Proliferation of lymph node T cells. T cells were purified from lymph nodes of *mkk7*$^{+/-}$*rag1*$^{-/-}$ and *mkk7*$^{-/-}$*rag1*$^{-/-}$ chimeric mice and activated (10^5 T cells/well) with soluble anti-CD3ϵ mAb (0.5 μg/ml), soluble anti-CD3ϵ (0.1μg/ml) plus anti-CD28 (20 ng/ml) Abs in the presence or absence of recombinant murine IL-2 (50 U/ml), Con A (2 μg/ml), or PMA (10 ng/ml) plus Ca^{2+} ionophore (100 ng/ml) (PMA+Iono). Mean proliferation ($\pm$ SD) was determined in triplicate at 48 h after a 12-h pulse with [^{3}H]thymidine. It should be noted that the surface marker phenotype and activation status of T cells to be stimulated were similar in cells of both genotypes; i.e., there was no bias in responder T cell populations. One result representative of five independent experiments is shown.

MKK7 Is a Negative Regulator of B Cell Proliferation. SAPK/JNK activation has been implicated in signal transduction 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$^+$sIgM$^+$, and CD19$^+$sIgM$^+$sIgD$^+$ B cells in the bone marrow, and normal populations of B220$^+$sIgM$^+$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, IgG2b, IgG3, and IgA were also comparable in *mkk7*$^{-/-}$*rag1*$^{-/-}$ and *mkk*$^{+/-}$*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*-deficient cells were stimulated with the intact anti-IgM Ab, suggesting that the hyperproliferation is not due to impaired negative signals mediated via FcγRIIB (not shown). The proliferation of *mkk7*-deficient B 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 MMK7 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 targeting 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. BMMCs of both genotypes showed similar expression levels of c-Kit (SCF-R) and the IgE receptor (not shown), two markers characteristic of mature BMMCs (37). Thus, loss of MKK7 expression does not prevent the emergence and differentiation of BMMCs. However, the proliferation of *mkk7*$^{-/-}$ BMMCs 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 BMMCs, paralleling the hyperproliferation observed in *mkk7*-deficient thymocytes and B cells. Again, no significant differences between *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs 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 BMMCs revealed that expression levels of p46 and p56 SAPK/JNK isoforms, p38 MAPK, ERK1/ERK2, PKB/Akt, and actin were comparable in *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs (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*$^{-/-}$ BMMCs, leading to a concomitant increase in cyclinD1 expression (Fig. 6 A). Interestingly, whereas expression of c-Jun was comparable between *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs, expression of JunB, which has been shown to upregulate p16INK4a expression (38), was mark-

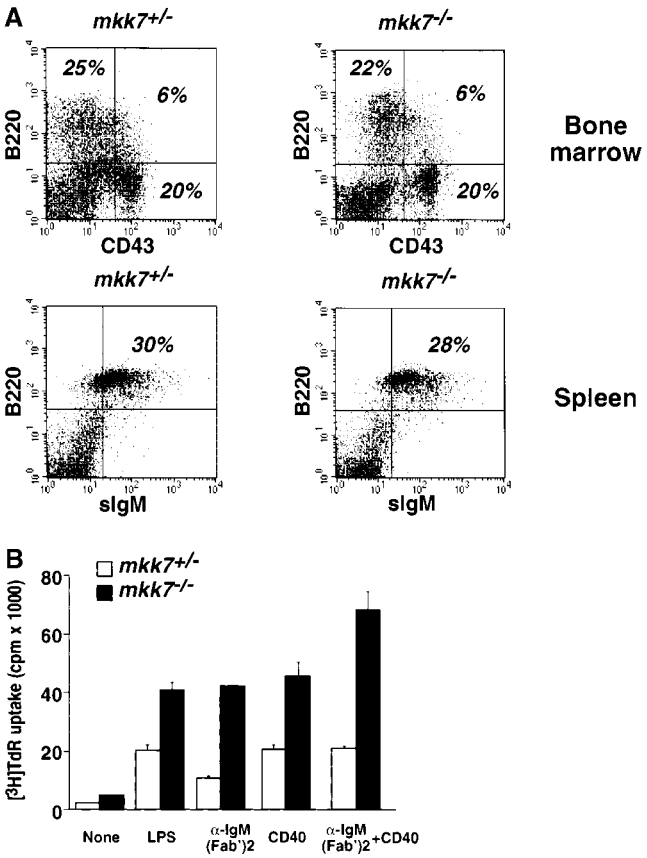

Figure 4. B cells hyperproliferate in the absence of MKK7. (A) Immunocytometric analysis of B cell populations in the bone marrow (top panels) and spleen (bottom panels) of *mkk7*$^{+/-}$ and *mkk7*$^{-/-}$ chimeric mice. Cells were isolated from 6-wk-old mice and double stained with anti-B220 and anti-CD43, or anti-B220 and anti-sIgM. Percentages of positive cells within a quadrant are indicated. (B) Activation of splenic B cells. Purified spleen cells (10^5/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 $(Fab')_2$ (5 μg/ml), anti-CD40 (5 μg/ml), or anti-IgM $(Fab')_2$ (5 μg/ml) plus anti-CD40 (5 μg/ml). Cells were labeled with [^{3}H]thymidine for the last 12 h of culture. The mean [^{3}H]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.

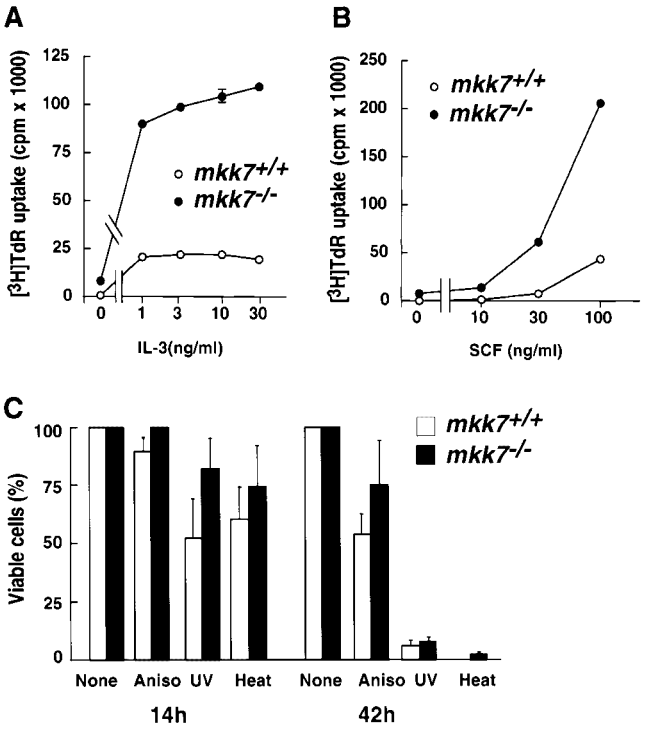

Figure 5. MKK7 regulates BMMC proliferation but not apoptosis. (A and B) Proliferation of *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs. *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs were incubated with increasing concentrations of (A) IL-3 or (B) SCF (c-Kit-Ligand). [^{3}H]Thymidine uptake was determined 24 h after cytokine addition. Similar results were obtained for five separate cultures from five mice of each genotype. (C) Induction of apoptosis in *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ BMMCs. 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*$^{-/-}$ BMMCs ($P > 0.1$) was also observed at various seeding numbers and in response to growth factor deprivation and osmotic shock (not shown).

edly decreased in *mkk7*$^{-/-}$ BMMCs. Expression of p27, another cell cycle inhibitor, was not affected by the absence of MKK7 (Fig. 6 A). However, it should be noted that loss of MKK7 does not always result in downregulation of p16INK4a expression, as p16INK4a expression appeared normal in B220^{+}IgM^{+} B cells after in vitro differentiation of *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ ES cells into B cells (Fig. 6 B, and data not shown). Thus, mutation of *mkk7* in BMMCs, but not in B cells, results in loss of expression of JunB and the cell cycle inhibitor p16INK4a and upregulation of cyclinD1.

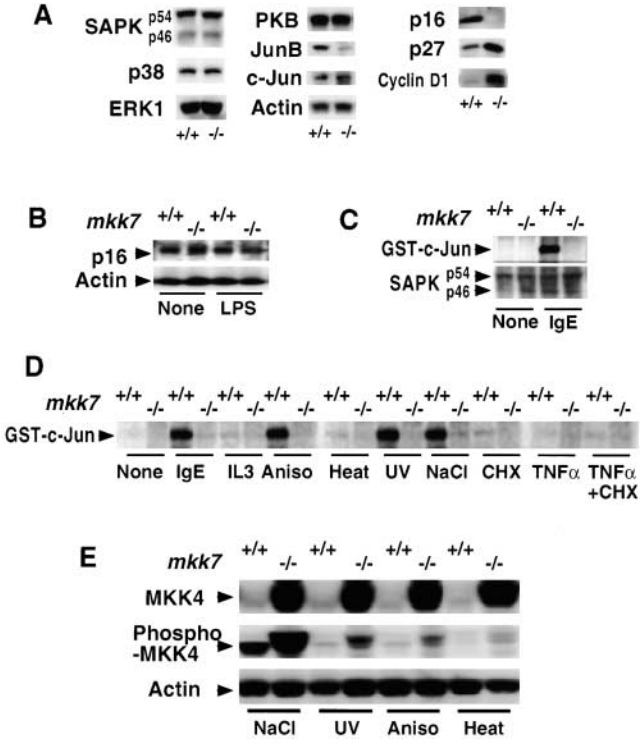

Figure 6. MKK7 is essential for SAPK/JNK activation in BMMCs. (A) Western blot analysis of expression levels of total p54 and p46 SAPK/JNK, p38 MAPK, ERK1, PKB/Akt, JunB, p16INK4a, p27, and cyclinD1 in *mkk7*$^{+/+}$ (+/+) and *mkk7*$^{-/-}$ (−/−) BMMCs. Actin is shown as a loading control. Total cell 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*$^{-/-}$ (−/−) B220^{+}IgM^{+} B cells. B cells were differentiated in vitro from *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ ES cells as described (reference 29). Total cell lysates of 10^6 cells were separated by SDS-PAGE and incubated with Abs specific for the indicated molecules. (C and D) SAPK/JNK activity 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 result 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/

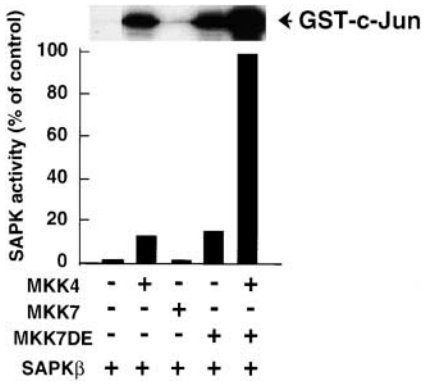

Figure 7. In vitro cooperation between MKK4 and MKK7 in SAPKβ/JNK3 kinase activation. Purified GST-SAPKβ/JNK3 was incubated with recombinant GST-MKK4 (wild-type MKK4), GST-MKK7 (wild-type MKK7), GST-MKK7DE (constitutively active MKK7), or GST-MKK4 plus GST-MKK7DE for 30 min. SAPKβ/JNK3 activation was measured by evaluating the amount of phosphorylated GST-c-Jun produced. One result representative of five independent experiments is shown for each lane.

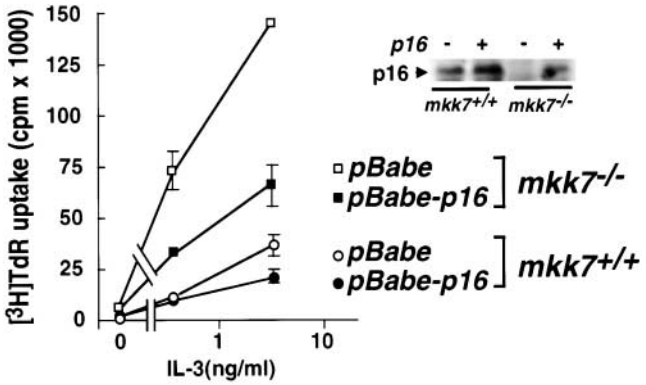

Figure 8. Ectopic expression of p16INK4a results in reduction of *mkk7*$^{-/-}$ BMMC hyperproliferation. *Mkk7* deficient and wild-type BMMCs were infected with empty virus (control) or p16INK4a expressing virus (p16) and selected for 1 wk with puromycin. After selection, cells were grown 3 more days without puromycin, and then, cell growth (thymidine uptake) was examined in the presence of different doses of IL-3. Expression of p16INK4a is shown (inset). One result representative of three independent experiments is shown.

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 results in hyperproliferation because reduced JunB expression and a loss of p16INK4a expression leads to upregulated cyclinD1 and accelerated cell cycle progression. To address whether p16INKa 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 p16INKa 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 hematopoeitic 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 osmolarity, toxins, ischemia/reperfusion injury in heart attacks, heat shock, anti-cancer drugs, ceramide, peroxide, 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, peroxide, 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 MKK → 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, SAPKβ/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*-deficient 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/Ca^{2+}-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 activation 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 required 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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