

Different Properties of SEK1 and MKK7 in Dual Phosphorylation of Stress-induced Activated Protein Kinase SAPK/JNK in Embryonic Stem Cells*

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Stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), belonging to the mitogen-activated protein kinase family, plays an important role in stress signaling. SAPK/JNK activation requires the phosphorylation of both Thr and Tyr residues in its Thr-Pro-Tyr motif, and SEK1 and MKK7 have been identified as the dual specificity kinases. In this study, we generated *mkk7*^{-/-} mouse embryonic stem (ES) cells in addition to *sek1*^{-/-} cells and compared the two kinases in terms of the activation and phosphorylation of JNK. Although SAPK/JNK activation by various stress signals was markedly impaired in both *sek1*^{-/-} and *mkk7*^{-/-} ES cells, there were striking differences in the dual phosphorylation profile. The severe impairment observed in *mkk7*^{-/-} cells was accompanied by a loss of the Thr phosphorylation of JNK without marked reduction in its Tyr-phosphorylated level. On the other hand, Thr phosphorylation of JNK in *sek1*^{-/-} cells was also attenuated in addition to a decreased level of its Tyr phosphorylation. Analysis in human embryonic kidney 293T cells transfected with a kinase-dead SEK1 or a Thr-Pro-Phe mutant of JNK1 revealed that SEK1-induced Tyr phosphorylation of JNK1 was followed by additional Thr phosphorylation by MKK7. Furthermore, SEK1 but not MKK7 was capable of binding to JNK1 in 293T cells. These results indicate that the Tyr and Thr residues of SAPK/JNK are sequentially phosphorylated by SEK1 and MKK7, respectively, in the stress-stimulated ES cells.

by many types of cellular stresses, including changes in osmolarity, heat shock, and UV irradiation, but also by serum, lysophosphatidic acid, and inflammatory cytokines (interleukin-1 β and tumor necrosis factor- α). The activated SAPK/JNK phosphorylates transcription factors c-Jun, Jun D, and activating transcription factor-2 to regulate gene expression for the stress response. Activation of SAPK/JNK requires the phosphorylation of Tyr and Thr residues located in a Thr-Pro-Tyr motif in the activation loop between VII and VIII of the kinase domain. The phosphorylation is catalyzed by the dual specificity kinases SEK1 (also known as MKK4) and MKK7 (SEK2), which are capable of catalyzing the phosphorylation of both Thr and Tyr residues *in vitro* (1, 2).

Targeted gene-disruption experiments in mice demonstrate that both SEK1 and MKK7 are required for embryonic development. *Sek1*^{-/-} embryos die between embryonic day 10.5 (E10.5) and E12.5 with impaired liver formation (3–5). Furthermore, we have recently reported that SEK1 is crucial for hepatocyte growth factor-induced activation of SAPK/JNK in developing hepatoblasts of mouse embryos. On the other hand, *mkk7*^{-/-} embryos die between E11.5 and E12.5 with similar impairment of liver formation and SAPK/JNK activation (6). These results clearly show that both SEK1 and MKK7 play indispensable roles in hepatoblast proliferation during mouse embryogenesis. Distinct biochemical properties between SEK1 and MKK7 may be critical for the indispensable roles of the two activators of SAPK/JNK *in vivo*.

In this regard, several *in vitro* experiments have shown that SAPK/JNK is activated synergistically by SEK1 and MKK7 (7–9). The synergistic activation may be related to the enzymatic properties of the two MAPKKs: SEK1 prefers the Tyr residue and MKK7 prefers the Thr residue of the MAPK. We have also reported that the synergistic activation of SAPK/JNK in response to stress signals is attenuated with a decreased level of its Tyr phosphorylation in *sek1*^{-/-} mouse ES cells that retain MKK7 at the same level as the wild-type cells (10).

The SAPK/JNK¹ is a member of the family of mitogen-activated protein kinase (MAPK). This MAPK is activated not only

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¹ The abbreviations used are: SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein ki-

nase; E, embryonic day; ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein kinase kinase; MAPKK, mitogen-activated protein kinase kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase kinase; ES, embryonic stem; Ab, antibody; mAb, monoclonal antibody; HA, hemagglutinin; Hyg, hygromycin resistance cassette; Neo, neomycin resistance cassette; dnSEK1, dominant-negative SEK1 mutant.

SAPK/JNK activation by UV irradiation and Fc ϵ -receptor stimulation was also attenuated in the *mkk7*^{-/-} mast cells. Despite the impaired SAPK/JNK activation in the *mkk7*^{-/-} cells, the expression of SEK1 was strongly up-regulated, and SEK1 protein was phosphorylated upon stimulation (11). Thus, both SEK1 and MKK7 seem to be required for the synergistic and functional activation of SAPK/JNK in a variety of mammalian cells.

To reveal the molecular mechanism of the synergistic activation of SAPK/JNK by SEK1 and MKK7 in living cells, we generated *mkk7*^{-/-} mouse ES cells by gene targeting, in addition to *sek1*^{-/-} ES cells, and further investigated the contribution of SEK1 and MKK7 to the activation and phosphorylation of the MAPK. Our present results clearly show that both SEK1 and MKK7 are required for the synergistic activation of SAPK/JNK in response to various stimuli in ES cells. Furthermore, we propose a sequential phosphorylation mechanism of SAPK/JNK by the two activators, SEK1 and MKK7, in the stress-stimulated living cells.

EXPERIMENTAL PROCEDURES

Generation of MKK7-deficient ES Cells—A 17-kbp DNA fragment of *mkk7* gene was isolated from a genomic 129/J mouse library. Targeting vector 1 contained a 585-bp short arm, a 6.7-kbp long arm, three loxP sequences, and a hygromycin resistance cassette (Hyg) in antisense orientation to *mkk7* transcription. Targeting vector 2 contained a 709-bp short arm, a 5.2-kbp long arm, and a neomycin resistance cassette (Neo) in antisense orientation to *mkk7* transcription. The linearized targeting vector 1 was electroporated into E14K ES cells. ES cell colonies resistant to hygromycin (0.2 mg/ml; Invitrogen) were screened for homologous recombination by PCR (30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, for 40 cycles) using primers specific for *mkk7* genomic sequences and Hyg as described below. Next, the *mkk7*^{+hyg} ES cells were transfected with Cre recombinase expression vector, and cell colonies sensitive to hygromycin were screened for deletion of the region containing exons 4–13 and Hyg by PCR. The linearized targeting vector 2 was electroporated into *mkk7*^{+del} ES cells. Retargeted ES cell colonies resistant to G418 (0.3 mg/ml; Invitrogen) were screened for homologous recombination by PCR using primers specific for *mkk7* genomic sequences and Neo as described below. As a result, two *mkk7*^{neo/del} clones (001 and 002) were independently obtained. Both clones lack MKK7 completely, and they are henceforth referred to as *mkk7*^{-/-} ES cells in this manuscript. Specific primer sets used were 5'-GCC AAA ACA CGG AGT GCT GG-3' and 5'-ATG TGA CCA GGC AGG AGT GG-3' for wild-type (+) allele, 5'-TTA AGG CAA CTG GCA GAG-3' and 5'-AGC TGA CTC TAG AGC TTG-3' for hyg allele, 5'-ATC TGC CTG TAG CAT GCC-3' and 5'-ACT CCA AAC ACC TCC CAC-3' for del allele, 5'-GGA TGT GGA ATG TGT GCG AG-3' and 5'-AGC TGG AAC CAC GCG CAA TGT GAG-3' for neo allele. Recombinant ES cell clones were confirmed by Southern blotting of *Xba*I-digested genomic DNA hybridized to a 530-bp 3'-flanking probe.

Plasmids—Plasmids that express FLAG-tagged SEK1, MKK7 γ 2, and HA-tagged JNK1 were constructed as described previously (10). The cDNA encoding FLAG-tagged SEK1 kinase dominant-negative mutant (dnSEK1) by substituting Lys-129 with Arg (K129R) was cloned into mammalian expression vector pCMV5. The cDNAs encoding SAPK/JNK1 mutants Ala-Pro-Phe (APF), Ala-Pro-Tyr (APY), and Thr-Pro-Phe (TPF) were constructed by Kunkel method using the following three primers: 5'-GGA ACG AGT TTT ATG ATG GCG CCT TTT GTA GTG ACT CGC TAC TAC AGA GCA CC-3' for APF mutant, 5'-GGA ACG AGT TTT ATG ATG GCG CCT TAT GTA GTG ACT CGC TAC TAC AGA GCA CC-3' for APY mutant, and 5'-GGA ACG AGT TTT ATG ATG GCG CCT TTT GTA GTG ACT CGC TAC TAC AGA GCA CC-3' for TPF mutant.

Antibodies—Antibodies against SAPK/JNK1 (C-17 and FL), MKK7/MEK7 (T-19), and SEK1/MEK4 (C-20) were purchased from Santa Cruz Biotechnology, Inc. Anti-phospho-SAPK/JNK (9251), and anti-phospho-SEK1 (9151) Abs were from Cell Signaling Technology. Anti-phospho-Tyr (PY20) and anti-phospho-Thr-Pro (P-Thr-Pro-101) mAbs were from BD Transduction Laboratories and Cell Signaling Technology, respectively. Anti-FLAG (M2) Ab and anti-HA affinity matrix were purchased from Sigma-Aldrich Co. and Roche Diagnostics, respectively. Rat anti-SEK1 (KN-001) and anti-MKK7 (KN-004) mAbs applicable to immunoprecipitation and immunoblotting were prepared in our laboratory as described previously (10).

Transfection—ES cells were plated at 2×10^6 cells onto a 60-mm dish and transfected 1 day later with 8 μ g of plasmid DNA using LipofectAMINE 2000 (Invitrogen). The cells, after being cultured for 1 day, were transferred to four 35-mm dishes and cultured for another day. Human embryonic kidney 293T cells were plated at 1×10^6 cells onto a 35-mm dish and transfected 1 day later with 4 μ g of plasmid DNA using LipofectAMINE 2000 (Invitrogen). The cells were stimulated and subjected for the assays of SAPK/JNK activity, immunoprecipitation, and immunoblotting.

Assay of SAPK/JNK Activity—ES cells were plated at 1.5×10^6 cells per 35-mm dish and cultured overnight. As chemical and physical stresses, the cells were stimulated by sorbitol (0.5 M; Wako), anisomycin (3 μ g/ml; Sigma), nocodazole (0.5 μ g/ml for 1 h; Sigma), UV light (1 kJ/m²), and heat shock (44 °C for 10 min). SAPK/JNK proteins were immunoprecipitated at 4 °C for 2 h using the anti-SAPK/JNK polyclonal Ab (C-17; Santa Cruz Biotechnology, Inc.). The SAPK/JNK activity in the precipitated fractions was measured with glutathione S-transferase-c-Jun as an *in vitro* substrate in the presence of 60 μ M [γ -³²P]ATP as described previously (12, 13).

Immunoprecipitation and Immunoblotting—To detect the phosphorylation of Tyr and Thr residues in the Thr-Pro-Tyr motif of endogenous SAPK/JNK, ES cells were plated at $\sim 2 \times 10^7$ cells onto a 150-mm dish and mixed with 2 ml of a lysis buffer (20 mM HEPES, pH 7.4, 1% Nonidet P-40, 10 mM NaCl, 0.05% 2-mercaptoethanol, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 100 μ M Na₃VO₄, 20 μ g/ml of leupeptin, 50 mM NaF, and 1 mM benzamide). The cell lysates were incubated with anti-SAPK/JNK Ab and protein A-Sepharose (Pharmacia) at 4 °C for 2 h, and the immuno-complexes were washed several times with the lysis buffer. The samples were analyzed by SDS-PAGE and immunoblotting. Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with anti-phospho-Tyr, phospho-Thr, and SAPK/JNK Abs. The bands were visualized by SuperSignal West Pico chemiluminescent substrate for the development of immunoblots using a horseradish peroxidase-conjugated second Ab according the manufacturer's instructions (Pierce). Endogenous SEK1 and MKK7 were immunoprecipitated with anti-SEK1 (KN-001) and anti-MKK7 (KN-004) mAbs and detected with anti-SEK1 (C-20) and anti-MKK7 (T-19) Abs, respectively.

All experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

RESULTS

Generation of MKK7-deficient ES Cells—To examine the role of MKK7 in SAPK/JNK regulation, we generated *mkk7*^{-/-} ES cells using two targeting vectors in the process of *mkk7*^{+/+}, *mkk7*^{+hyg}, *mkk7*^{+del}, and *mkk7*^{neo/del} construction as follows (Fig. 1). First, the exons 4–13 region of *mkk7* gene was deleted from one allele using targeting vector 1 and Cre recombinase (Fig. 1A). Second, another allele of *mkk7* was disrupted by replacing the site of activating phosphorylation in exon 9 with neomycin resistance cassette using targeting vector 2 (Fig. 1B). As a result, two clones (001 and 002) of *mkk7*^{neo/del} ES cells that completely lack 48-kDa MKK7 were independently obtained (Fig. 1, C and D). Thus, the null-mutant *mkk7*^{neo/del} clones are used as *mkk7*^{-/-} ES cells in this study.

Impaired Stress-induced SAPK/JNK Activation in MKK7-deficient ES Cells—To examine the role of MKK7 in stress-induced activation of SAPK/JNK, *mkk7*^{-/-} ES cells were incubated under various conditions, together with *sek1*^{-/-} and wild-type cells. Fig. 2 shows the time courses of SAPK/JNK activity in response to a protein synthesis inhibitor (A, 3 μ g/ml of anisomycin), heat shock (B, 44 °C for 10 min), UV irradiation (C, 1 kJ/m²), and hyperosmolar stress (D, 0.5 M sorbitol) in the two MAPKK mutant and wild-type ES cells. These stresses markedly stimulated SAPK/JNK activity in wild-type ES cells. Such stimulation, however, was severely impaired in both MAPKK mutant cells, although the cells contained SAPK/JNK at the same level as wild-type cells (see Fig. 4C). Thus, the stress-induced full activation of SAPK/JNK seems to require both SEK1 and MKK7 in ES cells.

The impairment of SAPK/JNK activation observed in the two

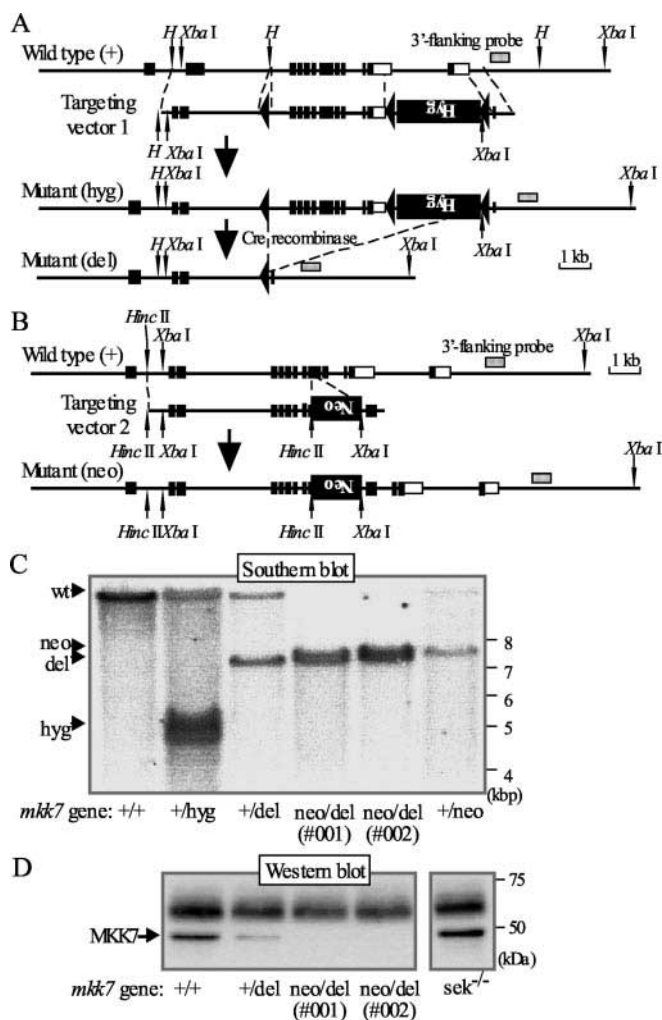


FIG. 1. Targeted gene disruption of murine *mkk7*. *A* and *B*, partial restriction map of murine genomic *mkk7* sequences and construction of targeting vectors 1 and 2. Targeting vector 1 (*A*) and 2 (*B*) contain a hygromycin resistance gene (Hyg) plus three loxP sequences (triangles) and a neomycin resistance gene (Neo), respectively. The 14 exons of *mkk7* are shown as boxes, and MKK7-coding regions as filled boxes. The genomic *mkk7* 3'-flanking probe used for Southern blotting is indicated as gray boxes. The predicted structures of targeted alleles are shown as *hyg*, *del*, and *neo*, respectively. Restriction enzymes used for the vector construction (*H*, *Hind*III, and *Hinc*II) and genomic Southern blotting (*Xba*I) are indicated by arrows. *C*, genomic analysis of ES cells. Genomic DNAs isolated from wild-type *mkk7*^{+/+}, *mkk7*^{+/hyg}, *mkk7*^{+/del}, *mkk7*^{+/neo}, and *mkk7*^{neo/del} E14K ES cells were digested with *Xba*I and analyzed by Southern blotting using the 3'-flanking probe. Molecular weight markers and *mkk7* alleles (*wt*, 13 kb; *hyg*, 5 kb; *del*, 7.3 kb; *neo*, 7.8 kb) are indicated by bars and arrows, respectively. *D*, Western blot analysis of 48-kDa MKK7 in ES cells. MKK7 was immunoprecipitated and immunoblotted with anti-MKK7 mAb and polyclonal Ab, respectively. The position of 48-kDa MKK7 is indicated by an arrow. No MKK7 was detected in the two independent clones of *mkk7*^{neo/del} ES cells (001 and 002). The amount of MKK7 in *sek1*^{-/-} ES cells was comparable with that in wild-type cells.

MAPKK mutant cells was further investigated with the different concentrations of sorbitol. As shown in Fig. 2*E*, the concentration-dependent activation curve of SAPK/JNK had a very steep upstroke at ~0.15 M sorbitol in wild-type ES cells. Interestingly, such steep activation of SAPK/JNK was markedly attenuated in *mkk7*^{-/-} and *sek1*^{-/-} ES cells without significant change in the half-maximum effective concentration of sorbitol. Impairment of SAPK/JNK activation in the two MAPKK mutant cells was also observed in other stress signals, including a microtubule-disruptive reagent, nocodazole, and genotoxic

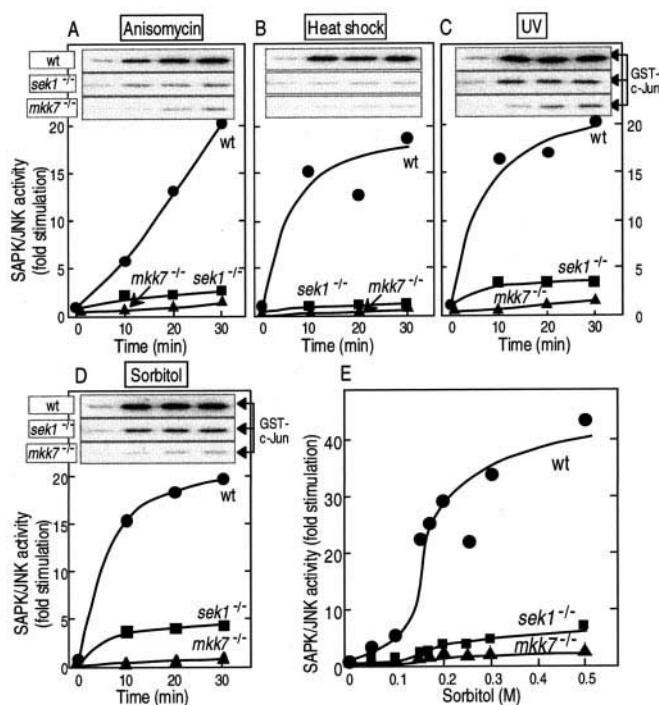


FIG. 2. Time courses of stress-induced activation of SAPK/JNK in wild-type, *sek1*^{-/-}, and *mkk7*^{-/-} ES cells. Wild-type, *sek1*^{-/-}, and *mkk7*^{-/-} ES cells were stimulated with anisomycin (*A*, 3 μ g/ml), heat shock (*B*, 44 $^{\circ}$ C for 10 min), UV (*C*, 1 kJ/m²) or sorbitol (*D*, 0.5 M and *E*, the indicated concentrations) for the indicated times or 30 min (*E*). Cell lysates were prepared from the stimulated cells and immunoprecipitated with an anti-SAPK/JNK (C-17) Ab. SAPK/JNK activity in the precipitated fractions was measured with glutathione *S*-transferase-*c*-Jun as a substrate in the presence of [³²P]ATP as described under "Experimental Procedures." The insets of *A*–*D* show the ³²P-phosphorylated glutathione *S*-transferase-*c*-Jun, and the activity is expressed as the -fold stimulation compared with the control level observed in wild-type ES cells without the stress. The data shown are representative of three independent experiments.

stresses, such as etoposide and arabinofuranosyl-cytosine (data not shown).

SEK1 and MKK7 Are Indispensable for Stress-induced SAPK/JNK Activation in ES Cells—The above results in Fig. 2 suggest that SEK1 and MKK7 synergistically contribute in the stress-induced stimulation of SAPK/JNK in ES cells. To elucidate the qualitative difference of the two MAPKKs, SEK1 and MKK7 expression vectors were transfected into *sek1*^{-/-} or *mkk7*^{-/-} ES cells (Fig. 3). SEK1 could rescue the impaired SAPK/JNK activation in response to UV irradiation and heat shock in *sek1*^{-/-} ES cells (Fig. 3*A*). However, MKK7 isoforms α 1, γ 1, and γ 2, could not restore the SAPK/JNK activation in *sek1*^{-/-} ES cells. On the other hand, MKK7 α 1 and γ 1, but not SEK1, could rescue the impaired SAPK/JNK activation in response to heat shock, anisomycin, and nocodazole in *mkk7*^{-/-} ES cells (Fig. 3*B*). These results clearly show that SEK1 and MKK7 serve different functions in the stress-induced SAPK/JNK activation in ES cells.

Properties of SEK1- and MKK7-induced Phosphorylation of SAPK/JNK—It has recently been reported that the phosphorylation of Thr and Tyr residues in the Thr-Pro-Tyr motif of SAPK/JNK is required for the full activation of the MAPK and that SEK1 and MKK7 preferentially phosphorylate the Tyr and Thr residues, respectively, *in vitro* (7–9). Therefore, we examined the stress-induced phosphorylation state of endogenous SAPK/JNK in *sek1*^{-/-} and *mkk7*^{-/-} ES cells together with wild-type cells. The three cell types were stimulated with 0.5 M of sorbitol (Fig. 4, lanes 2, 6, and 10), 1 kJ/m² of UV irradiation (lanes 3, 7, and 11), and 3 μ g/ml of anisomycin (lanes 4, 8, and

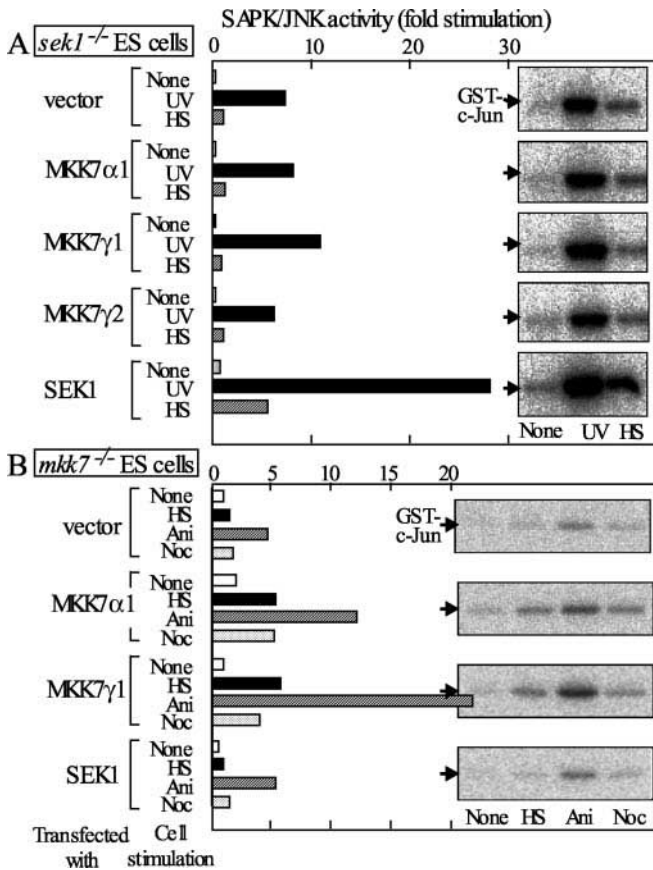


FIG. 3. Effects of SEK1 or MKK7 expression on stress-induced SAPK/JNK activation in *sek1*^{-/-} and *mkk7*^{-/-} ES cells. The two mutant *sek1*^{-/-} (A) and *mkk7*^{-/-} (B) ES cells were transfected with MKK7 α 1, γ 1, γ 2, or SEK1 expression vector. The transfected ES cells, after being cultured for 24 h, were stimulated with UV (1 kJ/m²) and further incubation for 25 min, heat shock (HS, 43 °C for 30 min), anisomycin (Ani, 10 μ g/ml for 30 min), or nocodazole (Noc, 0.5 μ g/ml for 1 h). Cell lysates were prepared and immunoprecipitated with an anti-SAPK/JNK (C-17) Ab. SAPK/JNK activity in the precipitated fractions was measured as described in Fig. 2.

12). The endogenous SAPK/JNK was immunoprecipitated and analyzed by immunoblotting with phospho-specific Abs. In a series of the present experiments, these ES cells expressed almost the same amounts of SAPK/JNK (Fig. 4C). The existence of stress-induced Thr and Tyr phosphorylation within the Thr-Pro-Tyr motif of SAPK/JNK in wild-type cells could be detected with anti-phospho-Thr-Pro and anti-phospho-Tyr Abs, respectively (Fig. 4, A and B, lanes 10–12). Interestingly, Thr but not Tyr phosphorylation was almost completely abolished in *mkk7*^{-/-} cells (Fig. 4, A and B, lanes 2–4) in which stress-induced phosphorylation of SEK1 was retained at the same level as wild-type cells (Fig. 4, D and E, lanes 5 and 6). On the other hand, the Tyr phosphorylation of SAPK/JNK was greatly impaired in *sek1*^{-/-} cells (Fig. 4B, lanes 6–8) in accordance with our previous report (10). Surprisingly, the Thr phosphorylation was also markedly attenuated in *sek1*^{-/-} cells (Fig. 4A, lanes 6–8), although this mutant contained the same amount of 48-kDa MKK7 as wild-type cells (Fig. 1D). These results indicate that SEK1 phosphorylates the Tyr residue of SAPK/JNK and that MKK7 preferentially phosphorylates the Thr residue of Tyr-phosphorylated SAPK/JNK rather than its non-phosphorylated form. In other words, MKK7-induced Thr phosphorylation requires the prior phosphorylation of SAPK/JNK at the Tyr residue by SEK1 in the stress-stimulated ES cells (see Fig. 8C).

Loss of Stress-induced Thr Phosphorylation of SAPK/JNK in

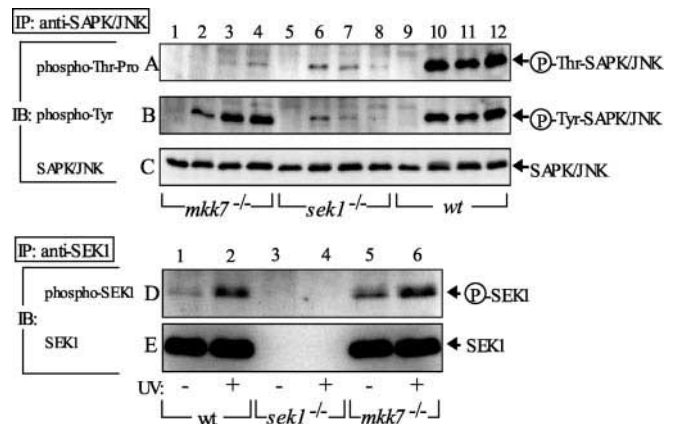


FIG. 4. Stress-induced Thr and Tyr phosphorylation of endogenous SAPK/JNK in wild-type, *sek1*^{-/-}, and *mkk7*^{-/-} ES cells. ES cells were stimulated with 0.5 M sorbitol (lanes 2, 6, 10), 1 kJ/m² of UV irradiation (lanes 3, 7, 11), and 3 μ g/ml of anisomycin (lanes 4, 8, 12) and further incubated at 37 °C for 25 min. Cell lysates were prepared, and endogenous SAPK/JNK and SEK1 were immunoprecipitated (IP) with anti-SAPK/JNK (C-17) polyclonal Abs (A–C) and anti-SEK1 (KN001) mAb (D and E), respectively. The Thr phosphorylation (A) and Tyr phosphorylation (B) of SAPK/JNK, together with phosphorylated SEK1 (D) were determined using anti-phospho Abs as described under “Experimental Procedures.” IB, immunoblots.

Cells Expressing a Kinase-dead Mutant of SEK1—In ES cells, SEK1 and MKK7 clearly contributed to the dual phosphorylation of SAPK/JNK in response to stress stimuli, and the MKK7-induced Thr phosphorylation seemed to require the prior Tyr phosphorylation by SEK1. Therefore, we further investigated the action of SEK1, which was supposed to be involved in the prior Tyr phosphorylation of SAPK/JNK. For the analysis, we used a human embryonic kidney cell line (293T) for transient transfection because of low efficiency of transfection into ES cells. HA-tagged JNK1 was co-expressed with FLAG-dnSEK1, which lacks kinase activity, in the cells using pCMV5 mammalian vectors. In a series of the experiments, the expression of HA-JNK1 was almost constant (Fig. 5C). Thr and Tyr phosphorylation of exogenous HA-JNK1 in response to 1 kJ/m² of UV irradiation was measured. UV irradiation induced the Thr and Tyr phosphorylation of HA-JNK1 (Fig. 5, A and B, lane 3); however, not only Tyr but also Thr phosphorylation was lost in the dnSEK1-expressing cells (Fig. 5, A and B, lane 4). These results clearly show that the stress-induced Thr phosphorylation of SAPK/JNK requires the prior Tyr phosphorylation by SEK1 in living cells.

Loss of Stress-induced Thr Phosphorylation of SAPK/JNK in Cells Expressing the No Tyr-phosphorylated Forms of SAPK/JNK—Next, we investigated the contribution of the Tyr residue in the Thr-Pro-Tyr motif of SAPK/JNK, phosphorylation of which was supposed to proceed before the Thr modification by MKK7 in the sequential phosphorylation. For the analysis, we constructed three kinds of HA-JNK1 mutants (APF, APY, and TPF), in which the Thr (T)-Pro (P)-Tyr (Y) motif was replaced with Ala (A), and Phe (F), respectively. The HA-JNK mutants and wild type were expressed in 293T cells by transfection, and the phosphorylation and kinase activity were measured. In a series of the experiments, the expression of HA-JNK1 was almost constant (Fig. 6C). The UV-induced Thr and Tyr phosphorylation and its ability to phosphorylate glutathione S-transferase-c-Jun as substrate could be detected in the wild-type HA-JNK1/TPY (Fig. 6, lane 1). Tyr phosphorylation of the HA-JNK/APY mutant was also detected (Fig. 6B, lane 3), although its kinase activity was completely lost (Fig. 6D, lane 3). Interestingly, the Thr phosphorylation could not be detected in the HA-JNK1/TPF mutant, in which the Tyr residue was re-

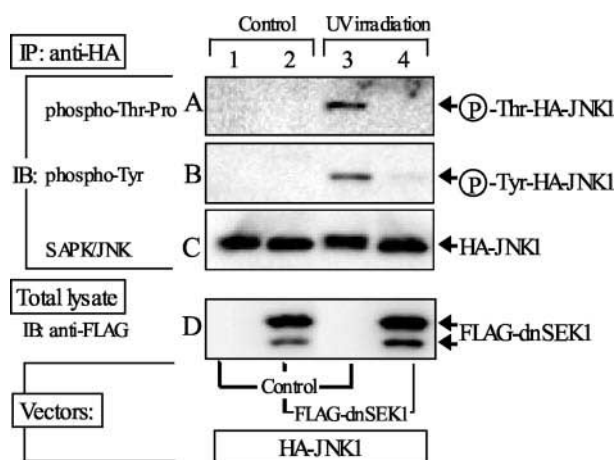


FIG. 5. Effects of SEK1 inhibition on stress-induced Thr and Tyr phosphorylation of SAPK/JNK in 293T cells. 293T cells were transfected with 1 μ g of pCMV5/HA-JNK1, together with (lanes 2 and 4) or without (lanes 1 and 3) 1 μ g of pCMV5/FLAG-dnSEK1. The transfected 293T cells, after being cultured for 24 h, were stimulated with 1 kJ/m² of UV irradiation and incubated for 25 min (lanes 3 and 4). Cell lysates were prepared, and HA-JNK1 was immunoprecipitated (IP) with anti-HA affinity matrix. The Thr phosphorylation (A) and Tyr phosphorylation (B) of HA-JNK1 were determined using anti-phospho Abs. HA-JNK1 (C) and FLAG-dnSEK1 (D) were determined using anti-SAPK/JNK (FL) and anti-FLAG (M2) Abs, respectively. IB, immunoblots.

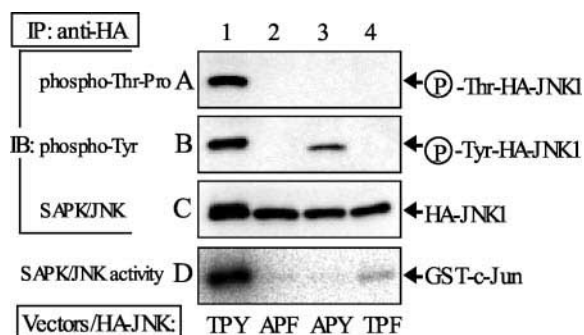


FIG. 6. Effects of amino acid replacement of the TPY motif on the stress-induced Thr and Tyr phosphorylation of SAPK/JNK in 293T cells. 293T cells were transfected with 1 μ g of TPY-wild type (lane 1), APF- (lane 2), APY- (lane 3), and TPF- (lane 4) mutant forms of HA-JNK1 expression vectors. The transfected 293T cells, after being cultured for 24 h, were stimulated with 1 kJ/m² of UV irradiation and further incubated for 25 min. Cell lysates were prepared and immunoprecipitated (IP) with anti-HA affinity matrix. The Thr phosphorylation (A) and Tyr phosphorylation (B) of HA-JNK1 were determined using anti-phospho Abs. HA-JNK1 (C) was determined using anti-SAPK/JNK (FL) polyclonal Abs. The SAPK/JNK activity (D) was measured as described under "Experimental Procedures." IB, immunoblots.

played by Phe (Fig. 6A, lane 4). These results clearly show that the stress-induced Thr phosphorylation of SAPK/JNK requires the phosphorylated Tyr residue in living cells.

SAPK/JNK Interacts More Preferentially with SEK1 than with MKK7—To understand the molecular mechanism of the prior Tyr phosphorylation of SAPK/JNK by SEK1, we examined the association of SAPK/JNK with SEK1. HA-JNK1 was co-expressed with FLAG-SEK1 and FLAG-MKK7 γ 2 in 293T cells using pCMV5 mammalian vectors (10). The transfected cells were stimulated with the protein synthesis inhibitor anisomycin. The cell lysates were immunoprecipitated with anti-HA affinity matrix and analyzed for the phosphorylation of SEK1 using anti-phospho-SEK1 and anti-FLAG Abs. The expression of HA-JNK1 was almost constant (Fig. 7C). The different ratios of FLAG-SEK1 and FLAG-MKK7 γ 2 expression vectors induced varied expression levels of FLAG-SEK1 and

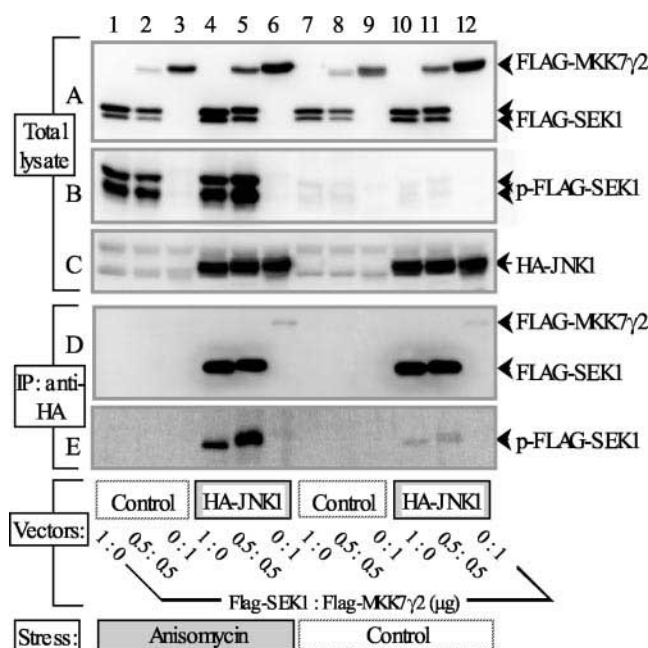


FIG. 7. Preferential association of SAPK/JNK with SEK1. 293T cells were transfected with different amounts (0, 0.5, or 1 μ g) of pCMV5/FLAG-tagged SEK1 and/or MKK7 γ 2 expression vectors, together with (lanes 4–6 and 10–12) or without (lanes 1–3 and 7–9) 1 μ g of pCMV5/HA-JNK1. The transfected 293T cells, after being cultured for 24 h, were stimulated with 3 μ g/ml of anisomycin (lanes 1–6) or not (lanes 7–12). Cell lysates were prepared (A–C) and immunoprecipitated (IP) with anti-HA affinity matrix (D and E). Expression of SEK1 plus MKK7 γ 2 (A), phosphorylated SEK1 (B), and HA-JNK1 (C) were determined using anti-FLAG (M2), anti-phospho-SEK1, and anti-SAPK/JNK (FL) Abs, respectively. Co-immunoprecipitated SEK1 plus MKK7 γ 2 (D) and phosphorylated SEK1 (E) were determined using anti-FLAG (M2) and anti-phospho-SEK1, respectively.

FLAG-MKK7 γ 2 proteins, but the sum of the expressed proteins was almost constant in each of the experiments (Fig. 7A). FLAG-SEK1 was phosphorylated in response to anisomycin in the presence and absence of HA-JNK1 (Fig. 7B). Interestingly, both phosphorylated and non-phosphorylated forms of FLAG-SEK1 could be coimmunoprecipitated with HA-JNK1; however, FLAG-MKK7 γ 2 was not (Fig. 7, D and E). The interaction between SEK1 and SAPK/JNK seemed to be comparable with that observed between JIP-1 and SAPK/JNK (data not shown). These results clearly show that SAPK/JNK interacts more preferentially with SEK1 than with MKK7 and suggest that the interaction might be responsible for the prior Tyr phosphorylation of SAPK/JNK by SEK1 (see Fig. 8C).

We also examined whether the above interaction is observable in ES cells that did not overexpress the HA-JNK1 and FLAG-SEK1/MKK7 γ 2. However, we could not detect their direct interaction in the native ES cells or in mouse tissues, including brain and liver (data not shown). This may be because of low expression levels of the endogenous proteins. Alternatively, endogenous SEK1 may localize near or exist as a non-associated form with SAPK/JNK in native cells, and there may be a molecular mechanism for releasing SEK1 rapidly after the phosphorylation of SAPK/JNK.

DISCUSSION

It has been reported in *in vitro* experiments that synergistic activation of SAPK/JNK requires the phosphorylation of both Thr and Tyr residues within the Thr-Pro-Tyr motif by the two different activators, SEK1 and MKK7 (7–9). Although the two MAPKKs are capable of catalyzing both phosphorylations, SEK1 prefers the Tyr phosphorylation and MKK7 prefers the Thr phosphorylation *in vitro* (Fig. 8A). In a previous study, we

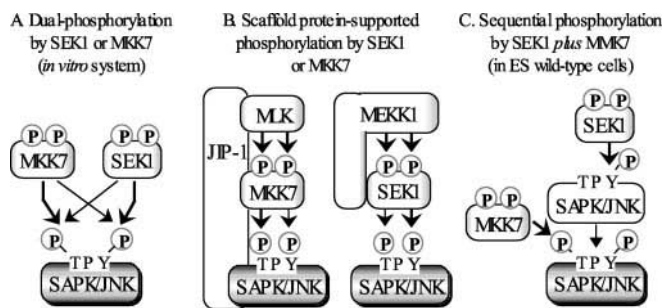


FIG. 8. Schematic description of SAPK/JNK phosphorylation by SEK1 and MKK7 under various conditions. A, synergistic activation of SAPK/JNK by the dual specificity kinase SEK1 or MKK7, which has been reported in *in vitro* conditions (7–9). B, activation of SAPK/JNK by SEK1 or MKK7 associated with their scaffold proteins, JIP-1 and MEKK1 (1, 2). C, synergistic activation of SAPK/JNK through sequential phosphorylation by SEK1 plus MKK7 in murine ES cells observed in the present study. See “Discussion” for further explanation. TPY, Thr-Pro-Tyr motif.

reported that SEK1 is essentially required for synergistic activation of SAPK/JNK in murine ES cells because the activation by various stresses was markedly attenuated in *sek1*^{-/-} murine ES cells (10). This attenuation was accompanied with a decreased level of the Tyr phosphorylation. In the present study, we also generated *mkk7*^{-/-} ES cells and compared the two mutant ES cells in terms of the activation and phosphorylation of SAPK/JNK. Our present results not only confirm the synergistic activation of SAPK/JNK reported previously but also indicate the unique properties of SEK1 and MKK7 in the stress-induced phosphorylation of the MAPK as follows.

First, MKK7 seemed to be a selective MAPKK for the synergistic activation and the Thr phosphorylation of SAPK/JNK. The mutant *mkk7*^{-/-} ES cells had a defect in synergistic SAPK/JNK activation in response to a variety of stimuli (Fig. 2). This defect could be selectively rescued by the introduction of MKK7 isoforms ($\alpha 1$ and $\gamma 1$) but not by SEK1 (Fig. 3B). Thr phosphorylation of SAPK/JNK observed in wild-type cells was almost completely abolished in *mkk7*^{-/-} ES cells (Fig. 4A, lanes 2–4; see also Fig. 8C). Second, the properties of MKK7, which preferentially catalyzes Thr phosphorylation of SAPK/JNK, seemed to be dependent on another MAPKK, SEK1, because the Thr phosphorylation was greatly impaired in *sek1*^{-/-} ES cells, which retain MKK7 expression (Fig. 4A, lanes 6–8). This idea was supported by the additional results as follows. 1) Inhibition of SEK1 by the expression of its dominant-negative form (dnSEK1) blocked Thr phosphorylation of SAPK/JNK in addition to Tyr modification. 2) The SAPK/JNK mutant (TPF), which lacks phosphorylatable Tyr residue, could not be phosphorylated at the Thr residue. 3) SEK1 could associate SAPK/JNK more preferentially than MKK7 and make a complex of SEK1 and SAPK/JNK without MKK7. Thus, we present a novel activation mechanism that SEK1-induced Tyr phosphorylation of SAPK/JNK is followed by additional Thr phosphorylation by MKK7 in stress-stimulated ES cells (Fig. 8C). In other words, MKK7 preferentially phosphorylates the Thr of Tyr-phosphorylated SAPK/JNK. On the other hand, SEK1 catalyzes Tyr phosphorylation of the MAPK in a manner independent on MKK7-induced Thr phosphorylation.

Tournier *et al.* have recently reported that the two MAPKKs, MKK7 and SEK1, differently contribute in various stress-induced activation of SAPK/JNK using primary murine embryo fibroblasts isolated from *sek1*^{-/-}, *mkk7*^{-/-}, and dual deficient mice (14). Their report shows that MKK7 is more important than SEK1 in the activation of SAPK/JNK by proinflammatory cytokines in murine embryo fibroblasts. SAPK/JNK activation

in response to UV and anisomycin was almost completely lost in *sek1*^{-/-} *mkk7*^{-/-} murine embryo fibroblasts, but approximately half stimulation of SAPK/JNK was retained in *sek1*^{-/-} or *mkk7*^{-/-} murine embryo fibroblasts. In contrast, SAPK/JNK activation in response to TNF α and IL-1 α was almost completely lost in *mkk7*^{-/-} cells, but 50% stimulation was observed in *sek1*^{-/-} cells. Thus, their results are somewhat different from ours observed in the single mutant of *sek1*^{-/-} or *mkk7*^{-/-} ES cells, where SAPK/JNK activation by various stimuli was greatly reduced. These differences may be caused by the specificity of cell types used. ES cells were derived directly from preimplantation embryos and maintained *in vitro* under undifferentiated conditions. Thus, the molecular mechanism of SAPK/JNK activation observable in ES cells may be considered a prototype in mammalian cells. In more differentiated cells, other cellular proteins, such as the JIP group of scaffold proteins, may regulate the protein interaction among SEK1, MKK7, and SAPK/JNK to alter the properties of the MAPKKs, resulting in the gain of dual-specificity kinase activity (Fig. 8B). However, overexpression of JIP1, -2, or -3 by cDNA transfection did not support this possibility (data not shown). Therefore, it will be critical to investigate the two phosphorylated states of the Thr-Pro-Tyr motif in endogenous SAPK/JNK and associated proteins to understand the molecular mechanism of synergistic activation of the MAPK in each type of cells.

Besides SAPK/JNK, other members of MAPK family, ERK and p38, have also two MAPKKs. ERK is activated by MKK1 and MKK2, and p38 is stimulated by MKK3 and MKK6 (15). Therefore, the presence of two MAPKKs is a common feature of mammalian MAPK-signaling pathways. Fleming *et al.* have reported that MKK3 and MKK6 have a strong preference for the phosphorylation of Tyr residue within the Thr-Gly-Tyr motif of p38 MAPK (9). Other MAPKKs have also unique biochemical properties. Therefore, ERK and p38 may be regulated cooperatively by two MAPKKs, as has been observed in SAPK/JNK. Studies in other MAPKK-deficient cells would be required for the elucidation of the functional significances of two activators in ERK and p38 MAPK-signaling pathways.

Recently, Ferrell *et al.* have proposed the interesting concept that SAPK/JNK cascade could, in principle, function as a sensitivity amplifier, which converts graded inputs into more switch-like outputs, allowing the cascade to filter out noise and yet still respond decisively to supra-threshold stimuli (16–19). They have shown in *Xenopus* oocytes that SAPK/JNK responds to physiological and pathological stimuli, such as progesterone and sorbitol, in an all-or-none manner (20). The activation of SAPK/JNK by the stimuli was graded at the level of a population of oocytes; however, at the level of an individual oocyte, the stimulatory response seemed to be switch-like. In the present study, we have also observed a very steep concentration-dependent response in the activation of SAPK/JNK by hyperosmolar stress (*i.e.* sorbitol) in murine ES cells (Fig. 2E). Furthermore, as described in the Introduction, our recent work showed that both SEK1 and MKK7 are required for full SAPK/JNK activation and hepatoblast proliferation in developing mice (6). This suggests that the all-or-none type MAPK activation also occurs in mammalian cells at an individual cell level only when the two MAPKKs are simultaneously activated. Therefore, this MAPK signaling should strictly proceed without errors, essentially through the two separated signals, one of which activates SEK1 and the other activates MKK7. Although the molecular mechanism whereby the two MAPKKs are simultaneously stimulated by various stress signals remains to be resolved, it is tempting to speculate that the existence of the two separated pathways leading to SAPK/JNK activation may

physiologically function as a fail-safe mechanism as proposed previously (10).

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