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Expression of Musashi1, a neural RNA-binding protein, in the cochlea of young adult mice

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Abstract

Musashi1 (Msi1) is an RNA-binding protein expressed in neural stem/progenitor cells, astroglial progenitor cells and astrocytes in the vertebrate central nervous system. We hypothesized that Msi1 is expressed in only some of the supporting cells in the cochlea, which could become hair cell progenitors under special circumstances after an injury. To observe this, we investigated Msi1 expression in young adult mouse cochlea by immunohistochemistry using monoclonal antibody against Msi1. Msi1 immunostaining was found in a variety of supporting cells but not in outer hair cells in the organ of Corti. Although an immunoreactive ring was found around the inner hair cells, it also seemed to originate from the supporting cells. We suppose that this wide expression of Msi1 in supporting cells indicates that those cells might have the potential to become hair cell progenitors if injured, but that some other mechanisms strictly inhibit this ability.

Keywords: Musashi1; Neural stem cell; Cochlea; Hair cells; Supporting cells; Regeneration

Musashi (MSI) was primarily isolated as the required molecule for asymmetric division of sensory organ precursor cells in Drosophila [7]. The MSI family is an evolutionarily conserved group of neural RNA-binding proteins, and Musashi1 (Msi1) is one of the mammalian homologues of MSI [10]. Msi1 is a neural RNA-binding protein expressed in neural stem/progenitor cells, astroglial progenitor cells and astrocytes in the central nervous system (CNS) [4,11]. Thus, Msi1 is considered one of the neural stem cell markers. Kaneko and colleagues speculated that this typical expression of Msi1 might be involved in the molecular mechanism required for reactivation or maintenance of quiescent glial cells to prepare for producing neural stem cells following stimulation [4]. It has also been demonstrated recently that Msi1 could be a Notch activator by translationally suppressing the synthesis of m-numb [3]. Notch lateral inhibition, which has a function in CNS development [8], is thought to play an important role in the development of the mammalian inner ear. After passing through terminal mitosis during development, some individual cells in the sensory epithelium of the cochlea begin to express Jagged2, one of the Notch ligands, and to activate Notch1 in the adjacent cells. The cells with activated Notch1 will develop as supporting cells, while Jagged2-expressing cells become hair cells [6]. To date, there is no evidence to suggest regeneration of hair cells in mammalian cochlea. However, mature birds are able to regenerate hair cells from supporting cells in their auditory sensory epithelium, when the loss of hair cells is induced by noise exposure or ototoxic drugs [13]. We attempted to determine potential hair cell progenitors in adult mammals. As the first step, we investigated Msi1 expression in young adult mouse cochlea by immunohistochemistry using a rat monoclonal anti-Msi1 antibody, clone 14H1 [4]. The antibody (14H1) recognizes amino acids 235–244 on Msi1 (LAPGY-TYQFP). This region is conserved in the *Xenopus*, mouse and human [4].

Experimental procedures involving animals reported in this study were performed according to the guidelines issued by the National Institute of Health, USA, and approved by the Animal Investigation Committee of Osaka University. Temporal bones were obtained from 8 pigmented CBA/N mice 32 days after birth (SLC, Shizuoka, Japan). Under deep anesthesia with diethyl ether, animals were fixed with 4% paraformaldehyde in 0.1 M phosphate–buffer

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(pH = 7.4). Tympanic bullae in the collected temporal bones were opened, and the specimens were immersed in fixative overnight at 4 °C, followed by decalcification with 8% ethylenediamine tetraacetic acid in fixative solution for 2 weeks at 4 °C. The decalcified specimens were embedded in Tissue-Tek O.C.T. compound (Miles Scientific, Elkhart, IN), then cryosections (12 μm) were cut. To block non-specific binding, we incubated the sections for 1 h in 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA) diluted in 0.1% Triton X in PBS (PBST). Subsequently, we incubated the sections at 4 °C for 16–20 h in 1:100 PBST dilution of rat anti-Msi1 monoclonal antibody ([4]: clone 14H1). The sections were then incubated for 1–2 h at room temperature with secondary antibody, 1:100 PBST dilution of fluorescein isothiocyanate-conjugated affinepure goat anti-rat IgG (H + L) (Jackson ImmunoResearch, WestGrove, PA). Both fluorescent and different interference contrast (DIC) images were recorded and analyzed with a Zeiss Confocal Laser Scan Microsope System LSM510. For negative controls, an absorption test was performed. Anti-Msi1 antibody (14H1) solution had been pre-incubated with the antigenic (blocking) peptide (5 μg/ml) at 4 °C overnight, which was used for the first antibody. Ventricular zones in the developing mouse spinal cord known to express Msi1 served as positive controls.

Msi1 immunoreactivity was observed in a variety of supporting cells in the organ of Corti (Figs. 1A and 2A,B). There was no immunoreactivity in outer hair cells (OHC), while immunoreactivity was significant in Deiters cells (DC), outer pillar cells (OPC), and inner pillar cells (IPC) (Figs. 2A,B). These supporting cells are firm, rich in microtubules, and constitute the framework of the organ of Corti. In the area outside the Deiters cells, the supporting cells such as Tectal cells (TC), Hensen cells (HC), Claudius cells (CC) and root cells (RC) were positively immunostained (Figs. 1A and 2A,B). The form of inner hair cell

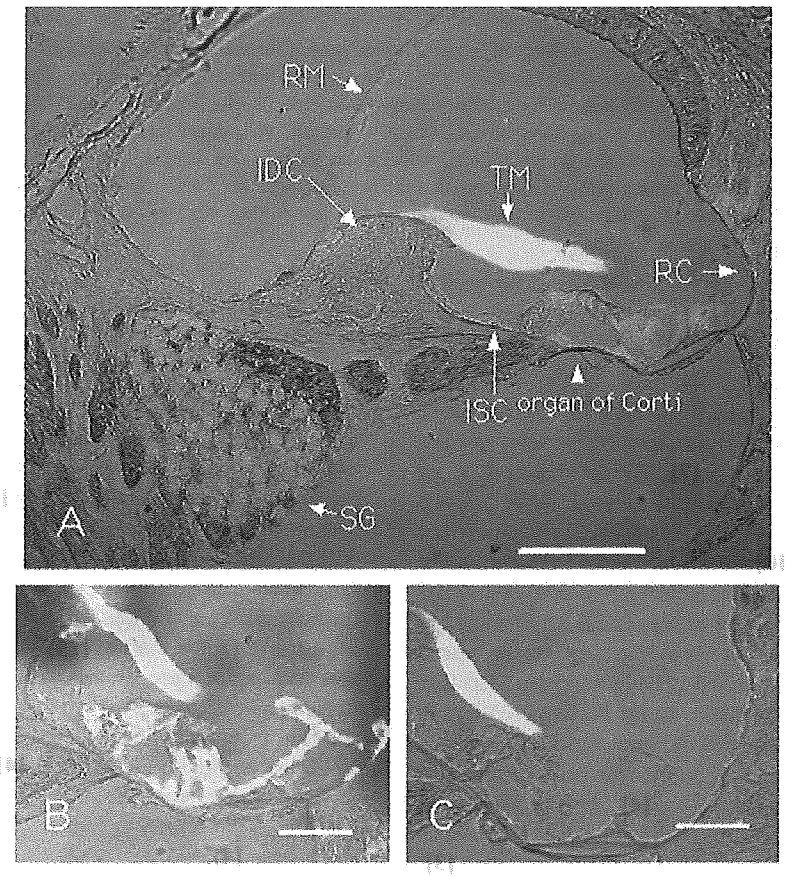

Fig. 1. Musahi1 (Msi1) immunoreactivity in the cochlea and verification of specificity of the antibody and immunostaining process under absorption test. (A) Msi1 immunoreactivity in the cochlea. The section of the cochlea under fluorescence illumination merged with the DIC image. In the organ of Corti, supporting cells around the hair cells demonstrated Msi1 immunoreactivity. The ISC and root cells (RC) were also positively stained by anti-Msi1 antibody. The neurons in the SG were immunostained by Msi1. Weaker reaction products, which were thought to be non-specific, were seen in the IDC and RM. Strong staining of the TM is also non-specific, because it persisted in the absorption test (Fig. 1C). Scale bar, 100 μm. (B) Msi1 immunostaining in the cochlea (positive control). The anti-Msi1 antibody (14H1) dilution was used for the first antibody. The following process was carried out together with the section used in the absorption test (Fig. 1C). Supporting cells of the organ of Corti and the TM were positively stained. Scale bar, 50 μm. (C) Result of the absorption test (negative control). Pre-incubation with antigenic peptide blocked the reaction in supporting cells of the organ of Corti. However, TM staining persisted, suggesting that this staining was non-specific. Scale bar, 50 μm.

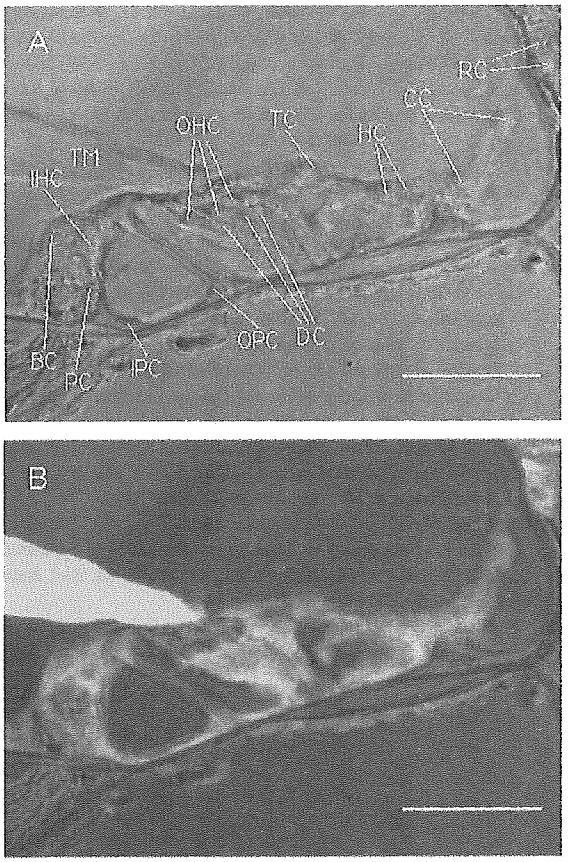

Fig. 2. Msi1 immunoreactivity in the organ of Corti. (A) Section of the organ of Corti under DIC optics. OHC, outer hair cell; IHC, inner hair cell; CC, Claudius cell; HC, Hensen cell; TC, tectal cell; DC, Deiters cell; OPC, outer pillar cell; IPC, inner pillar cell; PC, inner phalangeal cell; BC, inner border cell; and RC, root cell. Scale bar, 50 μm. (B) The same section under fluorescence illumination merged with DIC. The supporting cells surrounding OHC, such as OPC, DC, TC, HC, and CC were all positively stained by the anti-Msi1 antibody, but the OHCs were not stained at all. This typical mosaic pattern was also observed between the IHC and the supporting cells surrounding the IHCs. Indeed, immunoreactivity ringed the IHC, but it seemed to originate from the IPC, the inner PC, and the inner BC, which are all supporting cells. Positive immunostaining of RC could be seen in this figure as well as in Fig. 1A. Scale bar, 50 μm.

(IHC) is the flask–type, surrounded by supporting cells such as inner border cells (BC), inner phalangeal cells (PC) and inner pillar cells (IPC) (Figs. 2A,B). Similar to the outer hair cell area, the immunoreactive ring around the IHC seemed to originate from these supporting cells and did not appear to involve the IHCs themselves (Figs. 2A,B). The inner sulcus cells (ISC) adjacent to the inner border cells were also positively stained by anti-Msi1 antibody (Fig. 1A). The neurons in the spiral ganglion (SG) were immunostained by Msi1 (Fig. 1A). Weaker reaction products were seen in the interdental cells (IDC) and Reissner's membrane (RM) (Fig. 1A). However, RM and IDC were also faintly stained in the negative controls (data not shown). There was strong staining of the tectorial membrane (TM) (Figs. 1A,B). We considered this non-specific immunostaining, because Msi1 is an RNA binding protein and the TM does not include any cell components. Indeed, positive immunostaining of TM persisted in the negative controls, while staining of the supporting cells disappeared completely (Fig. 1C).

Msi1 is expressed in a variety of supporting cells of the organ of Corti but not in the hair cells themselves. While there have not been any reports confirming hair cell regeneration after injury in the cochleae of postnatal mammals, some reports have indicated this possibility. For example, cells with microvillar tufts resembling immature stereociliary bundles were detected in the cicatricial epithelium of former outer hair cell regions in young rats after drug-induced damage [9]. The replacement of hair cells was observed after laser beam irradiation in cultured organs of Corti from embryonic and neonatal mice [5]. The authors speculated that new cells moved from the inner sulcus region into the lesion site following laser microbeam irradiation to IHC, in cultured organs of Corti from neonatal mice. Cells with a morphological character indicative of immature hair cells were observed just adjacent to a few of these lesion sites, and one such cell was even thought to have arisen through renewed proliferation based on the autoradiographic data using ^{3}H-thimidine. These findings may indicate that the inner sulcus cells prepare hair cell progenitors, which are restricted only during the embryonic and neonatal periods. Our findings indicated that Msi1 was expressed in various kinds of supporting cells of adult mice, including inner sulcus cells. Based on this Msi1 expression, we suggest that those Msi1-positive supporting cells might have the potential to become hair cells after injury, but that some other mechanisms strictly inhibit this potential. For example, Chen and Segil reported that $p27^{Kip1}$, an inhibitor of cell cycle progression continued to be expressed in the supporting cells of mature mice. They speculated that it contributed to the quiescent state of the organ of Corti [2].

Msi1 immunoreactivity was found in the root cells of the cochlea outside the organ of Corti. The expression of Msi1 in mice, as well as being expressed in neural tissues, has been confirmed in granulosa cells of the ovaries, Sertoli cells of the testes, and small intestines of rodents [1,12]. Sertoli cells are known to provide germ cells with factors necessary for survival and differentiation such as androgen binding protein and physical support [12]. Based on their morphological features, root cells are thought to function in ion transport and fluid regulation. We thought that root cells and other Msi1-positive cells such as Sertoli cells, share certain similarities as epithelial cells functioning in transport and secretion.

In conclusion, we demonstrated for the first time that RNA binding protein Msi1 is expressed in supporting cells of mouse cochlea. The relationship between Msi1 expression in supporting cells of the adult mouse cochlea and their quiescent state of regeneration remains to be clarified.

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Stress Induces Mitochondria-mediated Apoptosis Independent of SAPK/JNK Activation in Embryonic Stem Cells*

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SAPK/JNK, which belongs to the family of mitogen-activated protein kinase (MAPK), is activated by many types of cellular stresses or extracellular signals and is involved in embryonic development, immune responses, and cell survival or apoptosis. However, the physiological roles of SAPK/JNK in the signaling of stress-induced apoptosis are still controversial. To evaluate the precise function, SAPK/JNK-inactivated mouse embryonic stem (ES) cells were generated by disrupting genes of the MAPK activators, SEK1 and MKK7. Although SAPK/JNK activation by various stresses was completely abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells, apoptotic responses including DNA fragmentation and caspase 3 activation still occurred normally, which displays a sharp contrast to *apaf1*$^{-/-}$ ES cells exhibiting profound defects in the mitochondria-dependent apoptosis. These normal apoptotic responses without SAPK/JNK activation were also observed in fibroblasts derived from *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells. Instead, interleukin-1β (IL-1β)-induced IL-6 gene expression was greatly suppressed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ fibroblasts. These results clearly show that SAPK/JNK activation is responsible for the inflammatory cytokine-induced gene expression but not essentially required for the mitochondria-dependent apoptosis at least in ES or fibroblast-like cells, which are prototypes of all cell lineages.

Apoptosis or programmed cell death is critical for many biological events such as embryonic development, immune responses, and tissue homeostasis in multicellular organisms. In mammalian cells, apoptotic signaling cascades can be divided into two broad categories: the intrinsic (mitochondria-dependent) and the extrinsic (death receptor-mediated) pathways. The initiation of mitochondria-dependent pathway requires a change in the organella membrane permeability that is prevented by anti-apoptotic molecules such as Bcl-2 and Bcl-X_L and promoted by pro-apoptotic molecules including Bax and Bak, and the permeability change results in the release of mitochondrial proteins. One of the released proteins, cytochrome *c*, associates with Apaf1 and caspase 9 to activate the effector caspase 3 (1, 2). Cellular stresses such as UV irradiation and heat shock mediate apoptosis through the mitochondria-dependent pathway (3). However, upstream signaling that regulates the pro-apoptotic molecules remains to be elucidated.

Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK),[1] which belongs to the family of mitogen-activated protein kinase (MAPK), is activated not only by many types of cellular stresses including UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin, and tunicamycin but also by inflammatory cytokines, interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α). The activated SAPK/JNK phosphorylates a number of substrates including the c-Jun component of the activator protein-1 transcription factor to regulate gene expression for the stress responses. Activation of SAPK/JNK requires the dual phosphorylation of Tyr and Thr residues located in a Thr-Pro-Tyr motif of the MAPK, and two kinases, SEK1 (also known as MKK4) and MKK7 (SEK2), are responsible for the phosphorylation (4–6). SEK1 and MKK7 preferentially phosphorylate the Tyr and Thr residues of SAPK/JNK, respectively (7). Interestingly, the Tyr phosphorylation by SEK1 is sequentially followed by the Thr phosphorylation by MKK7 in stress-stimulated mouse embryonic stem (ES) cells (8, 9).

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 and massive apoptosis (10, 11). We have recently shown that SEK1-mediated SAPK/JNK pathway downstream TNF-α receptor 1 participates in embryonic hepatoblast proliferation and survival via a pathway different from NF-κB-induced anti-apoptosis. On the other hand, *mkk7*$^{-/-}$ embryos die between E11.5 and 12.5 with similar defects in liver formation. These results indicate that SAPK/JNK activation mediated through SEK1 *plus* MKK7 plays indispensable roles in hepatoblast proliferation and survival during mouse embryogenesis (12).

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[1] The abbreviations used are: SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase kinase; ES, embryonic stem; MEF, mouse embryonic fibroblast; IL, interleukin; TNF-α, tumor necrosis factor α; E, embryonic day; ERK, extracellular signal-regulated kinase.

However, the physiological role of SAPK/JNK activation in cell survival and apoptosis is still controversial, being suggested to have an anti-apoptotic, pro-apoptotic, or no function in these processes (13). Mice lacking both JNK1 and JNK2 (*Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$) die around E11 with defective neural tube morphogenesis and altered apoptosis (14, 15). These results assign both pro- and anti-apoptotic functions to JNK1 and JNK2 in the development. It also appears that the SAPK/JNK pathway functions in a manner dependent on the types of cells and stimulus, and its different components can sometimes play opposing roles in apoptosis. The most convincing evidence to date that shows that the involvement of SAPK/JNK activation in pro-apoptotic function comes from *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ and *mkk4*$^{-/-}$ *mkk7*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Both *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ and *mkk4*$^{-/-}$ *mkk7*$^{-/-}$ MEFs exhibited profound defects in stress-induced apoptosis (16, 17). Furthermore, it has been reported that active JNK causes the release of apoptogenic factors such as cytochrome *c* and Smac from isolated mitochondria in a cell-free system (18, 19). These results strongly indicate that the SAPK/JNK activation directly regulates mitochondria-dependent apoptosis in pro-apoptotic direction.

To evaluate the exact role of SAPK/JNK activation in mitochondria-dependent apoptosis, we here utilized mouse ES cells in terms of the following advantages. 1) ES cells are a prototype of all cell lineages and can be differentiated into MEF-like cells with retinoic acid. 2) ES cells do not express death receptors including Fas and TNF-α receptor 1 but have stress-induced mitochondria-dependent apoptotic pathway. 3) The molecular mechanism of SAPK/JNK activation is well characterized in ES cells. The present results clearly show that SAPK/JNK activation is not required for the stress-induced mitochondria-dependent apoptosis in ES and MEF-like cells. Instead, we found that IL-1-induced IL-6 gene expression was greatly impaired in MEF derived from *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—The murine ES cell line E14K (wild type) was maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and leukemia inhibitory factor as described previously (20). The generation of *apaf1*$^{-/-}$ ES cells was described previously (3). *Sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells were newly generated as described in Fig. 2. MEF-like cells are prepared from ES cells by culture with 10 μM retinoic acid for 14 days without leukemia inhibitory factor. For thermotolerance, cells were incubated at 44 °C for 10 min and further cultured for 6 h.

Antibodies against SAPK/JNK1 (C-17 and FL), p38 (C-20), ERK2, and Bax were purchased from Santa Cruz Biotechnology, Inc. Anti-phospho-SAPK/JNK (number 9251) and anti-phospho-p38 (number 9211) antibodies were from New England BioLabs, Inc. Anti-mitochondrial heat shock protein 70 was from Affinity BioReagents. Rat anti-SEK1 (KN-001) and anti-MKK7 (KN-004) antibodies applicable to immunoprecipitation and immunoblotting were prepared in our laboratory (8). SB203580 and SP600125 were from Calbiochem and Biomol, respectively. Adenovirus-encoding Cre recombinase (number 1748) was from DNA Bank, BioResource Center, RIKEN (Ibaraki, Japan) (21).

Generation of ES Cells Lacking Both SEK1 and MKK7—sek1 and *mkk7* genes were disrupted as shown in Fig. 2*a*. Steps 1 and 2 were done by using a *sek*1 (neomycin)-targeting vector and gene-dosage effect, respectively (20). Steps 3 and 4 were performed by using a *mkk7* (loxP-hygromycin)-targeting vector and an adenovirus-encoding Cre recombinase, respectively (9, 21). Step 5 was done by using a novel *mkk7* (puromycin)-targeting vector as shown in Fig. 2*a*.

Assay of SAPK/JNK Activity—ES cells were plated at 1.5 × 10^6 cells/35-mm dish and cultured for overnight. The cells were stimulated by anisomycin (Sigma, 5 μg/ml), cisplatinum (Sigma, 50 μM), etoposide (Sigma, 2 μg/ml), thapsigargin (Sigma, 200 nM), tunicamycin (Sigma, 10 μg/ml), UV (1 kJ/m^2, UV Stratalinker 1800, Stratagene), and heat shock (44 °C for 20 min). SAPK/JNK proteins were immunoprecipitated at 4 °C for 2 h using the anti-SAPK/JNK antibody (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 μM [γ-^{32}P]ATP (8, 9). The amount of the precipitated SAPK/JNK that had been monitored by immunoblotting with the anti-SAPK/JNK (FL) antibody was almost constant in a series of the present experiments.

Immunoprecipitation and Immunoblotting—ES cells (2 × 10^6 cells) were suspended at 4 °C in 0.2 ml of a lysis buffer consisting of 1% Nonidet P-40, 80 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 4 μg/ml aprotinin. The cell lysates were incubated for 30 min and centrifuged at 15,000 rpm for 15 min. The supernatants were analyzed by SDS-PAGE and immunoblotting. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with antibodies against anti-SAPK/JNK1, anti-ERK2, anti-p38, and anti-phospho-SAPK/JNK. The bands were visualized by SuperSignal West Pico chemiluminescent substrate for the development of immunoblots utilizing a horseradish peroxidase-conjugated second antibody according to the manufacturer's instructions (Pierce). For detection of phospho-p38, cells were lysed directly in Laemmli sample buffer and sonicated by Ultrasonic Disruptor (TOMY) followed by SDS-PAGE and immunoblotting. Endogenous SEK1 and MKK7 were immunoprecipitated with anti-SEK1 (KN-001) and anti-MKK7 (KN-004) and detected with anti-SEK1 (C-20) and anti-MKK7 (T-19) antibodies, respectively (8, 9).

DNA Fragmentation Assay—ES cells (2 × 10^6 cells), which had been attached to and detached from culture dishes after stimuli, were collected by means of incubation with trypsin/EDTA and centrifugation, respectively. The cells were mixed, washed twice with phosphate-buffered saline, and collected by centrifugation. After removing the supernatants, the cells were lysed in 0.33 ml of a buffer consisting of 5 mM Tris-HCl (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100. After centrifugation at 27,000 × *g* for 20 min at 4 °C to remove nuclei and insoluble fraction, the cell lysates were subjected to phenol extraction and ethanol precipitation for DNA purification. The precipitated DNA was suspended in 20 μl of H_2O and treated with 50 μg/ml RNase for 30 min at 37 °C. The DNA samples (10 μl) were subjected to electrophoresis on 2% agarose gels and visualized by a UV illuminator (22).

Assay of Caspase 3 Activity—ES cells were harvested as described above and washed twice with phosphate-buffered saline. The cells, after being resuspended in 50 μl of a buffer consisting of 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, were frozen in liquid nitrogen and thawed at 37 °C three times. The cell lysates (50 μg) were incubated at 37 °C for 1 h with 20 μM acetyl-Asp-Glu-Val-Asp α-(4-methyl-coumaryl-7-amide) (DEVD-MCA; Peptide Institute, Inc.) in the final volume of 50 μl in 20 mM Hepes-NaOH (pH 7.4), 2 mM dithiothreitol, and 10% glycerol. The reaction was terminated by adding 450 μl of ice-cold H_2O, and substrate cleavage leading to the release of free MCA was monitored (excitation 355 nm and emission 460 nm) at room temperature (22).

Sub-G_1 Assay—For flow-cytometric analysis, cells were first fixed with 70% ethanol and further incubated with 10 μg/ml RNase A at 37 °C for 1 h. The cells were stained in a solution (50 μg of propidium iodide/ml, 0.1% sodium citrate, and 0.1% Nonidet P-40) for 30 min at 4 °C. The apoptotic sub-G_1 population was determined by FACScan flow cytometer (BD Biosciences) with Cell Quest software (23).

Subcellular Fractionation—ES cells (1 × 10^7 cells) were harvested by cell scraper (SUMILON) and washed twice with phosphate-buffered saline. The cells, after being resuspended in 100 μl of buffer A consisting of 250 mM sucrose, 20 mM HEPES-KOH (pH 7.4), 10 mM KCl, 1.5 mM Na-EGTA, 1.5 mM Na-EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 2 μg/ml aprotinin, were homogenized 10 strokes by a 27-gauge syringe. After homogenization, cells were centrifuged at 600 × *g* for 10 min at 4 °C to remove nuclei, unbroken cells, and large debris. Supernatants containing mitochondria were transferred to a new tube and further centrifuged at 10,000 × *g* for 10 min at 4 °C. Mitochondrial pellets were washed in 100 μl of buffer A followed by centrifugation at 10,000 × *g* for 10 min at 4 °C and lysed in 1.5× Laemmli sample buffer. The samples (10 μl) were analyzed by SDS-PAGE, and immunoblotting was probed with the anti-Bax and mitochondrial heat shock protein 70 antibodies.

Northern Blotting—Total RNA was prepared from differentiated ES cells by TRIzol reagent (Invitrogen), separated by formamide agarose gel, and transferred to a Hybond-XL (Amersham Biosciences). IL-6 and β-actin were detected by Northern blotting using the specific DNA probes. The cDNA fragments corresponding to mouse IL-6 and β-actin were amplified using the following primers: IL-6, 5′-ATG AAG TTC CTC TCT GCA AGA GAC T-3′ and 5′-CAC TAG GTT TGC CGA GTA GAT CTC-3′, and β-actin, 5′-CAT CAC TAT TGG CAA CGA GC-3′ and 5′-ACG CAG CTC AGT AAC AGT CC-3′.

All of the experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproduc-

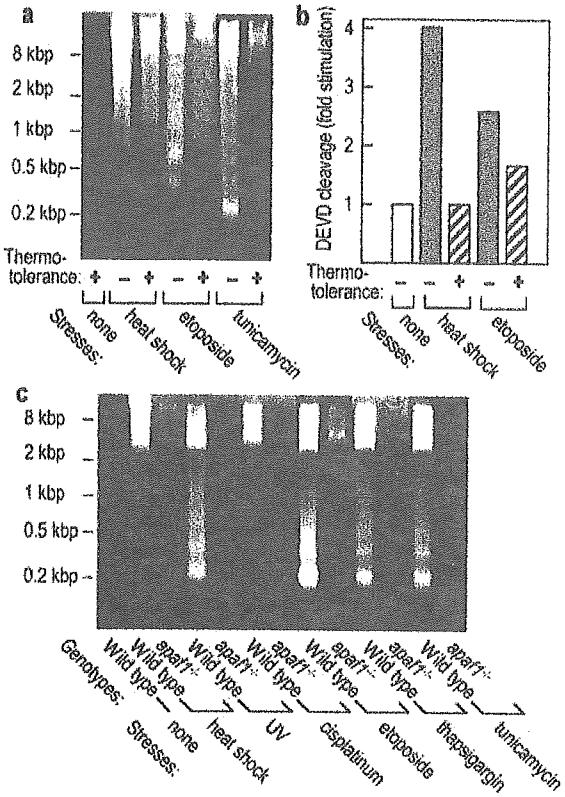

FIG. 1. **Characterization of stress-induced apoptosis in ES cells.** Wild-type ES cells were treated with (+) or without (−) heat shock at 44 °C for 10 min (*thermotolerance*), incubated for 6 h, and stimulated with the indicated stresses for 12 h. *a*, agarose-gel electrophoresis of DNA fragmentation. *b*, caspase 3 activation measured by DEVD cleavage. *c*, wild-type and *apaf1*$^{-/-}$ ES cells were stimulated with heat shock (44 °C for 20 min), UV (20 J/m^2), cisplatinum (50 μM), etoposide (2 μg/ml), thapsigargin (200 nM), or tunicamycin (10 μg/ml) and further incubated for 12 h. DNA fragmentation was measured by agarose-gel electrophoresis. The data shown are representative of three independent experiments.

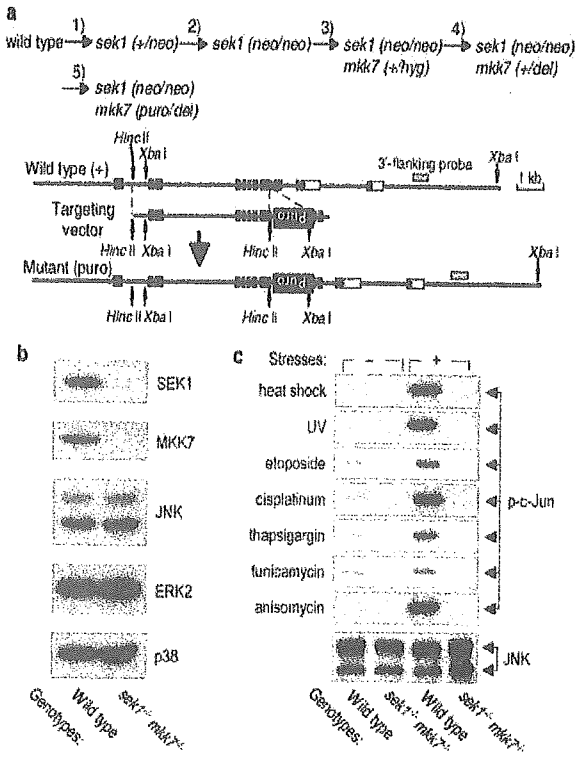

FIG. 2. **Targeted gene disruption of both *sek1* and *mkk7* in mouse ES cells.** *a*, flow chart of the disruption of *sek1* and *mkk7* genes and a novel *mkk7* (puromycin)-targeting vector. The genomic *mkk7* 3′-flanking probe used for Southern blotting is indicated as *gray boxes*, and the predicted structures of targeted alleles are shown as *puro*. Restriction enzymes used for the vector construction (HincII) and genomic Southern blotting (XbaI) are indicated by *arrows*. See "Experimental Procedures" for further explanation. *b*, Western blot analysis of SEK1, MKK7, JNK, ERK2, and p38 in wild-type and *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells. *c*, SAPK/JNK activity in wild-type and *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells. ES cells were stimulated with heat shock (44 °C for 20 min), UV (1 kJ/m^2 for 20 min), etoposide (2 μg/ml for 8 h), cisplatinum (50 μM for 8 h), thapsigargin (200 nM for 30 min), tunicamycin (10 μg/ml for 30 min), or anisomycin (5 μg/ml for 30 min). The amounts of JNK, ERK2, and p38 in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells were comparable with those in wild-type cells.

ible. Hence, most of the data shown are representative of several independent experiments.

RESULTS

Various Stresses Induce Apoptosis through Mitochondria in Mouse ES Cells—We first investigated the thermotolerance effect on apoptosis in ES cells, which protects cells against successive stress by the pretreatment of mild heat shock. For this mechanism, a recent study revealed that HSP70 and HSP27 induced by thermotolerance suppress mitochondria-dependent apoptosis by directly associating with Apaf1 and cytochrome *c* and blocking the assembly of a functional apoptosome (24–26). As shown in Fig. 1, various stresses could induce DNA ladder formation (Fig. 1*a*) and caspase 3 activation, which was measured by DEVD cleavage (Fig. 1*b*) in wild-type ES cells. However, these apoptotic responses were markedly attenuated by the thermotolerance treatment. To confirm the involvement of Apaf1 in the stress-induced apoptosis, ES cells lacking Apaf1 (*apaf1*$^{-/-}$) (3) were subjected to the same experiments. As shown in Fig. 1*c*, apoptosis in response to various stresses including heat shock, UV, cisplatinum, etoposide, thapsigargin, and tunicamycin was almost completely blocked in *apaf1*$^{-/-}$ ES cells. These results clearly show that the stress-induced apoptosis is mediated through mitochondria in ES cells.

Stress-induced SAPK/JNK Activation Is Impaired in ES Cells Lacking both MKK4 and MKK7—To examine the role of SAPK/JNK activation in the stress-induced and mitochondria-dependent apoptosis, ES cells lacking both MKK4 and MKK7 were generated. For generation of the null mutant, we constructed a novel *mkk7*-targeting vector containing puromycin-resistant cassette (Fig. 2*a*), and disrupted *sek1* and *mkk7* genes as described previously (9, 20). The mutant *sek1(neo/neo) mkk7(puro/del)* clones thus generated lack both SEK1 and MKK7 completely (Fig. 2*b*), and these clones are used as *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells in this study. As shown in Fig. 2*c*, not only stress-induced activation but also the basal level of SAPK/JNK was completely lost in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells.

Stress-induced Apoptosis Is Still Observed in sek1$^{-/-}$ *mkk7*$^{-/-}$ *ES Cells*—To examine the SAPK/JNK-inactivation effect, various apoptotic responses including caspase 3 activation, DNA fragmentation, and sub-G_1 population were further analyzed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells in comparison with wild-type and *apaf1*$^{-/-}$ ES cells (Fig. 3). Surprisingly, the apoptotic responses were still observed clearly in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells at the same levels as wild-type ES cells. In sharp contrast, such responses were almost completely abolished in *apaf1*$^{-/-}$

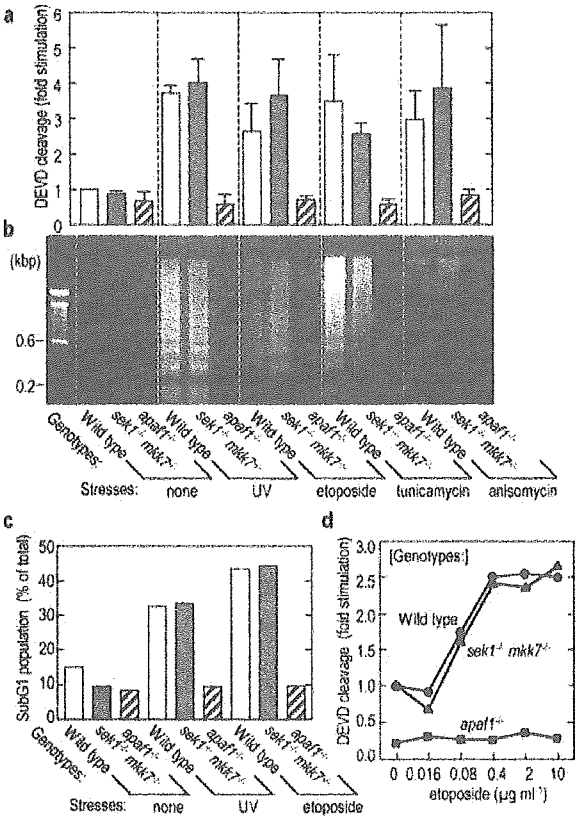

FIG. 3. **Stress-induced apoptosis in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ ES cells.** ES cells were stimulated with UV (20 J/m^2), etoposide (2 μg/ml), tunicamycin (10 μg/ml), or anisomycin (5 μg/ml) and further incubated for 12 h, and caspase 3 activation (*a*), DNA fragmentation (*b*), and sub-G_1 population (*c*) were measured. ES cells were stimulated with the indicated concentrations of etoposide, and caspase 3 activation (*d*) was measured.

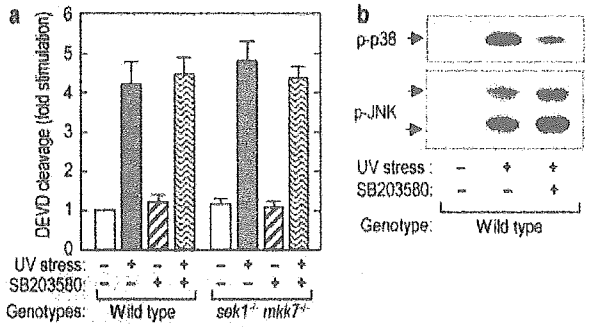

FIG. 4. **Effect of p38 inhibition on stress-induced apoptosis in ES cells.** *a*, ES cells were incubated with (+) or without (−) 10 μM SB203580 for 1 h, stimulated with UV (20 J/m^2), and further incubated for 12 h. UV-induced apoptosis was estimated by caspase 3 activation. *b*, ES cells were stimulated with UV (1 kJ/m^2 for 30 min) in the presence (+) or absence (−) of 10 μM SB203580, and active forms of p38 and JNK were detected by immunoblotting with anti-phospho-p38 and anti-phospho-JNK antibodies.

cells. The dose dependence and time course of etoposide-induced apoptosis were also the same between *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and wild-type ES cells (Fig. 3*d* and data not shown). Thus, the SAPK/JNK inactivation appears to exert no influence on the stress-induced apoptosis in ES cells.

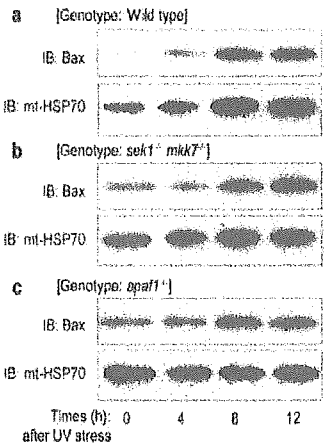

FIG. 5. **Stress-induced Bax translocation in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ ES cells.** ES cells were stimulated with UV (200 J/m^2) and further incubated for 4, 8, and 12 h. Mitochondrial membranes were prepared and immunoblotted (*IB*) with anti-Bax and anti-mitochondrial heat shock protein 70 (*mt-HSP70*) antibodies.

No Involvement of p38 Activation in Stress-induced Apoptosis in sek1$^{-/-}$ *mkk7*$^{-/-}$ *ES Cells*—Since it has been reported that another stress-responsive MAPK, p38, plays a pro-apoptotic role under certain conditions (27), we examined the effect of a p38 inhibitor (SB203580) on UV stress-induced apoptosis. As shown in Fig. 4, SB203580 markedly suppressed the phosphorylation of p38 but failed to inhibit the stress-induced apoptosis both in wild-type and *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells. These results indicate that p38 activation is not responsible for the stress-induced apoptosis in ES cells.

No Requirement of SAPK/JNK Activation for Bax Translocation in ES Cells—The above results shown in Figs. 1–3 suggest that SAPK/JNK activation is not required for mitochondria-dependent apoptosis in ES cells. We next measured the initiation step of mitochondria-dependent apoptosis, the translocation of Bax from cytoplasm to mitochondrial membranes, since it has been recently reported that SAPK/JNK activation potentiates Bax-dependent apoptosis in neuronal cells (28). Fig. 5 shows the time courses of the Bax translocation in response to UV irradiation in *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *apaf1*$^{-/-}$, and wild-type ES cells. There were no significant differences among the three types of ES cells. These results clearly show that SAPK/JNK activation is not required for the initiation of mitochondria-dependent apoptosis in ES cells.

Stress-induced Apoptosis Is Also Observable in sek1$^{-/-}$ *mkk7*$^{-/-}$ *MEF-like Cells*—Since it has recently been reported that SAPK/JNK is crucial for stress-induced apoptosis in MEF (16, 17), ES cells were treated with retinoic acid and induced to differentiate into MEF-like cells. As shown in Fig. 6, stress-induced apoptosis measured by DNA fragmentation was also observed at the same level between *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and wild-type MEF-like cells as had been observed in non-differentiated ES cells (see Fig. 3*b*). These results indicate that SAPK/JNK inactivation does not exert its influence on the stress-induced apoptosis in MEF-like cells as well as in ES cells.

IL-1-induced IL-6 Gene Expression Is Impaired in sek1$^{-/-}$ *mkk7*$^{-/-}$ *MEF-like Cells*—To understand the physiological role of SAPK/JNK activation in ES or MEF-like cells, we examined gene expression induced by an inflammatory cytokine, IL-1. As shown in Fig. 7*a*, IL-6 mRNA was induced at 1 and 6 h in wild-type cells. However, this induction was greatly inhibited in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells, which express IL-1 receptors. Furthermore, the IL-1-dependent IL-6 expression

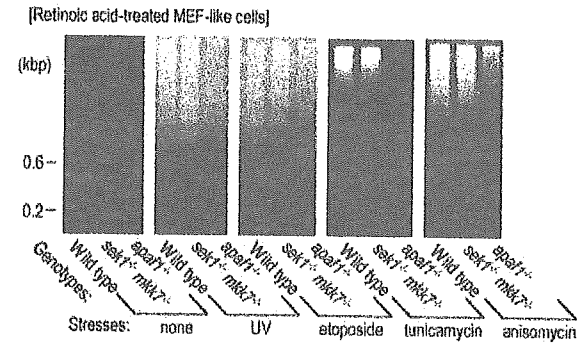

FIG. 6. **Stress-induced apoptosis in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ MEF-like cells.** ES cells were treated with 10 μM retinoic acid for 14 days and differentiated into MEF-like cells. The cells were stimulated UV (200 J/m^2), etoposide (20 μg/ml), tunicamycin (50 μg/ml), or anisomycin (50 μg/ml) and further incubated for 20 h. UV-induced apoptosis was measured by DNA fragmentation.

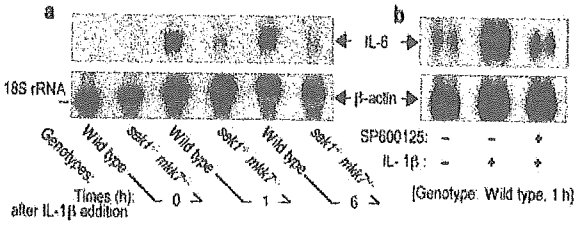

FIG. 7. **IL-1-induced IL-6 gene expression is impaired in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells.** *a*, wild-type and *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells were stimulated with 100 ng/ml IL-1β for 1 and 6 h. *b*, wild-type MEF-like cells were incubated with (+) or without (−) 50 μM SP600125 for 1 h and further stimulated with IL-1β for 1 h. Total RNAs were prepared and applied on agarose gel. IL-6 and β-actin mRNAs were detected by Northern blot analysis.

was greatly suppressed by adding the JNK inhibitor SP600125 in wild-type MEF-like cells (Fig. 7*b*). Thus, SAPK/JNK activation appears to play an important role in the inflammatory cytokine-induced gene expression rather than the apoptotic responses at least in ES or MEF-like cells.

DISCUSSION

In the previous study, we have reported that *sek1*$^{-/-}$ thymocytes are susceptible to Fas/CD95- and CD3-mediated apoptosis and that apoptosis is increased in *sek1*$^{-/-}$ hepatoblasts (11, 20). These results indicate that SAPK/JNK activation is involved in cell survival. However, it has been reported that *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ and *mkk4*$^{-/-}$ *mkk7*$^{-/-}$ MEFs are resistant to UV-induced apoptosis (16, 17). Furthermore, there are many other reports supporting the involvement of SAPK/JNK activation both in cell survival and in apoptosis. Thus, the role of SAPK/JNK in cell survival and apoptosis is still controversial (13). Our present results do not support the concept that "SAPK/JNK activation is required for the stress-induced and mitochondria-dependent apoptosis" (4, 16, 17), at least in ES and MEF-like cells, based on the following findings.

First, SAPK/JNK activation is completely dependent on the existence of both SEK1 and MKK7 in mouse ES cells in which stress-induced apoptosis is mediated through mitochondria (Fig. 1). The mutant *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells had a defect in the SAPK/JNK activation in response to a variety of stimuli (Fig. 2) but displayed normal stress-induced apoptosis as the same level as wild-type cells (Fig. 3). Another stress-responsive MAPK, p38, was not involved in the stress-induced apoptosis (Fig. 4). Second, Bax was capable of translocating into mitochondrial membranes in response to UV irradiation in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells as observed in the wild-type cells (Fig. 5). Third, the apoptosis in response to various stresses such as UV, etoposide, tunicamycin, and anisomycin occurred even in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells. Therefore, there must be a SAPK/JNK-independent signaling that is required for the initiation of stress-induced apoptosis in ES and MEF-like cells.

To examine the involvement of MKK7 in cell cycle and senescence, we have recently prepared *mkk7*$^{-/-}$ MEFs from mouse embryos. Interestingly, early passaged wild-type and *mkk7*$^{-/-}$ MEFs showed comparable extent and kinetics of cell death in response to UV irradiation; however, at late time points of culture (after getting cellular immortality), *mkk7*$^{-/-}$ MEFs displayed a resistance to the UV-induced apoptosis.[2] These results suggested that the resistance to UV response observed in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ and *mkk4*$^{-/-}$ *mkk7*$^{-/-}$ MEFs (16, 17) might have some relations to cellular immortality. Because SAPK/JNK activation is crucial for hepatoblast proliferation (12), the immortalized MEFs may lose the regulation of checkpoint in cell cycle and result in resistance to stress-induced apoptosis due to impaired G_1 growth arrest. Therefore, it will be interesting to find out whether re-expression of JNK1 plus JNK2 in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ or MKK4 plus MKK7 in *mkk4*$^{-/-}$ *mkk7*$^{-/-}$ MEFs can restore the apoptotic response to UV stress.

However, recent reports with *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mice clearly show that SAPK/JNK activation is required not only for anti-apoptosis but also for pro-apoptosis in the development of mouse fetal brain (14, 15). There was a reduction of cell death in the lateral edges of hind brain prior to neural tube closure. In contrast, there was increased apoptosis and caspase activation in the forebrain in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ mouse embryos. Thus, SAPK/JNK activation plays a pro-apoptotic role under some circumstances. SAPK/JNK activation may be involved in regulating apoptosis indirectly rather than directly.

SAPK/JNK is activated in T cells by co-stimulation with antigen and CD28 receptors and regulates the production of growth factor IL-2 and cell proliferation (29). Previously, we reported impaired CD28-mediated IL-2 production and proliferation in *sek1*$^{-/-}$ T lymphocytes (23). It has recently been reported that activation of wild-type CD8$^+$ T cells in the presence of different concentrations of SP600125 caused a dose-dependent inhibition of IL-2 production and cell proliferation (30). Thus, SAPK/JNK activation plays an important role in T cell functions. IL-1 is a pro-inflammatory cytokine in inflamed tissues, and the molecular mechanism of IL-1-induced gene expression will yield novel molecular targets for anti-inflammatory therapy. It has been reported that SAPK/JNK activation is required for the gene expression of a cytokine IL-6 and a chemokine IL-8 in a human epidermal carcinoma cell line by using overexpression of a catalytically inactive mutant or antisense RNA of SAPKβ (31, 32). These results indicate that IL-1-mediated activation of SAPK/JNK induces the phosphorylation of a Jun family of component(s) of activator protein-1, resulting in the gene expression of IL-6 and IL-8. In this study, we have also observed the impaired IL-1-mediated IL-6 gene expression both in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells and in SP600125-treated wild-type cells (Fig. 7). These results provide the first genetic link between SAPK/JNK activation and IL-6 gene expression using gene-disrupted cells and also indicate that SAPK/JNK activation provides a crucial and specific signal for immune responses. Thus, the molecular switch, SAPK/JNK activation, regulates cell proliferation and gene expres-

[2] T. Wada, N. Joza, H.-Y. M. Cheng, T. Sasaki, I. Kozieradzki, M. Nghiem, K. Bachmaier, T. Katada, H. Nishina, and J. M. Penninger, submitted for publication.

sion in embryonic development and immune responses rather than stress-induced apoptosis.

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Activation mechanism and physiological roles of stress-activated protein kinase/c-Jun NH_2-terminal kinase in mammalian cells

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ABSTRACT: Stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), which belongs to the family of mitogen-activated protein kinase (MAPK), is activated by many types of cellular stress or extracellular signals. Recent studies, including the analysis with knockout cells and mice, have led towards understanding the molecular mechanism of stress-induced SAPK/JNK activation and the physiological roles of SAPK/JNK in embryonic development and immune responses. Two SAPK/JNK activators, SEK1 and MKK7, are required for full activation of SAPK/JNK, which responds to various stimuli in an all-or-none manner in mouse embryonic stem (ES) cells. SAPK/JNK activation plays essential roles in organogenesis during mouse development by regulating cell proliferation, survival or apoptosis and in immune responses by regulating cytokine gene expression. Furthermore, SAPK/JNK is involved in regulation of mRNA stabilization, cell migration, and cytoskeletal integrity. Thus, SAPK/JNK has a wide range of functions in mammalian cells. (J Biol Regul Homeost Agents 2003; 17:)

KEY WORDS: *Stress, MAPK, SAPK/JNK, Apoptosis, Knock out, ES cells*

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MAP kinases (MAPKs) are evolutionary conserved signal-transducing enzymes involved in the regulation of many cellular events. Several MAPK groups have been identified in mammalian cells, including extracellular signal-regulated kinase (ERK), p38, ERK5, and SAPK/JNK. These MAPKs are activated by their specific MAPK kinases (MAPKKs): ERK by MEK1 and MEK2, p38 by MKK3 and MKK6, ERK5 by MEK5, and SAPK/JNK by SEK1 (also known as MKK4) and MKK7 (SEK2). These MAPKKs are also activated by various MAPKK kinases (MAPKKKs) such as Raf, MLK, MEKK1, TAK1, and ASK1. In this review, we focus on recent progress in the SAPK/JNK group of MAPK-signaling pathways in mouse embryonic stem (ES) cells, embryos, and adult mice (Fig. 1). It is becoming clear that SAPK/JNK activation regulates many cellular processes such as gene expression, cell survival and apoptosis (1-4).

Molecular mechanism of SAPK/JNK activation in ES cells

SAPK/JNK is activated not only by many types of cellular stress including changes in osmolarity, UV irradiation, heat shock, cisplatinum, etoposide, thapsigargin, and tunicamycin, but also by serum, lysophosphatidic acid (LPA), and inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor αβ (TNF-α). The activated SAPK/JNK phosphorylates a number of substrates including transcription factors, c-Jun, Jun D, and ATF-2, to regulate gene expression for stress responses. Activation of SAPK/JNK requires the dual 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 (5). The phosphorylation is catalyzed by the dual specificity kinases, SEK1 and MKK7, which are capable of catalyzing the phosphorylation of both Thr and Tyr residues.

Recent studies have shown that SEK1 has a preference for the phosphorylation of Tyr residue, while MKK7 for Thr residue of SAPK/JNK *in vitro* and that both phosphorylation results in the synergistic activation of SAPK/JNK (Fig. 2A) (6-8). Strong support for this activation mechanism has been obtained from studies of SEK1- and MKK7-gene disruption in ES cells. The severe impairment of SAPK/JNK activation observed in $mkk7^{-/-}$ ES cells was accompanied with a loss of the Thr-phosphorylation of SAPK/JNK, without marked reduction in its Tyr-phosphorylated level. On the other hand, Thr-phosphorylation of SAPK/JNK in $sek1^{-/-}$ ES cells was also attenuated, in addition to a decreased level of its Tyr-phosphorylation. These results indicate that the Tyr and Thr residues of SAPK/JNK are sequentially phosphorylated by SEK1 and MKK7, respectively, in stress-stimulated ES cells (Fig. 2B) (9, 10).

Involvement of "scaffold proteins" has also been reported for the correct proceeding of SAPK/JNK-

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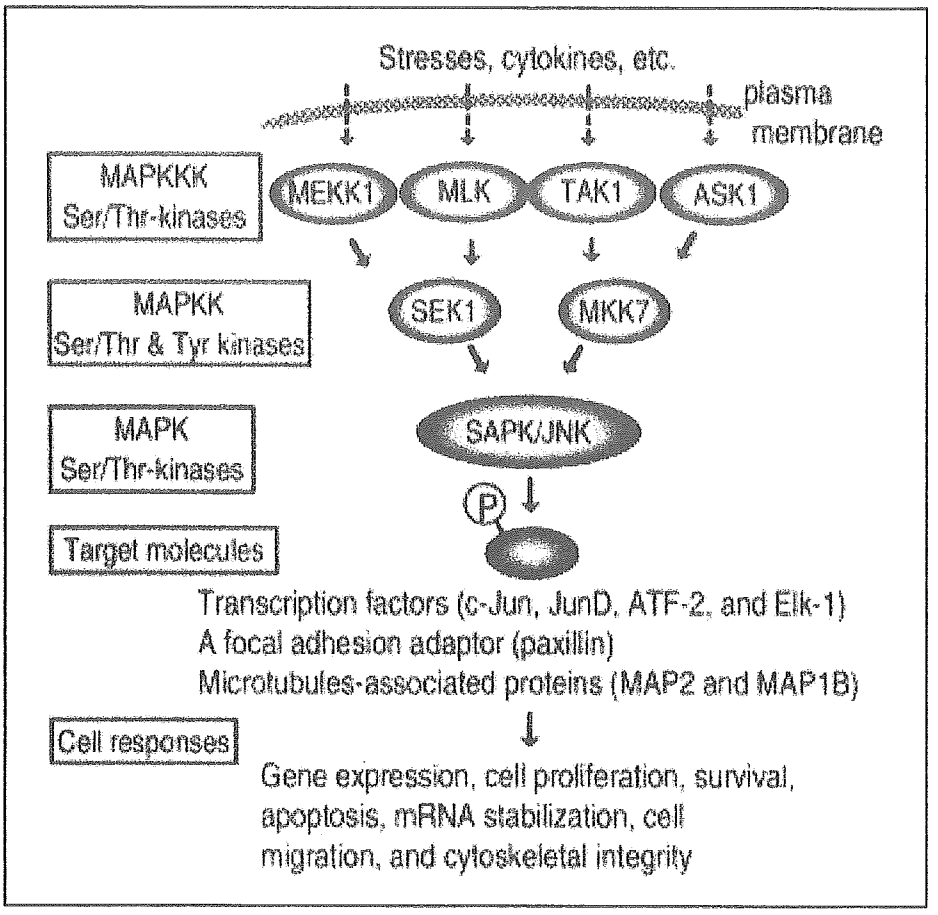

Fig. 1 - SAPK/JNK-signaling pathways involved in a variety of cell responses. SAPK/JNK is activated by extracellular stimuli including stresses and cytokines through kinase cascades. Activated MAPKKKs such as MEKK1, MLK, TAK1, and ASK1 transmit the signal to two MAPKK, SEK1 and MKK7. SAPK/JNK activated by SEK1 and/or MKK7 phosphorylates target molecules such as c-Jun, paxillin, and MAP2 to regulate a wide range of cell functions.

signaling pathway. The scaffold proteins, JIP-1, JIP-2, JIP-3, JSAP1, and JLP, organize the components of SAPK/JNK cascade to secure SAPK/JNK-signaling specificity in mammalian cells (11-15). JIP-1, JIP-2, and JIP-3 bind to SAPK/JNK, MKK7, and mixed-lineage protein kinases (MLKs). JSAP1 is an alternatively spliced variant of JIP3, however, associates with SAPK/JNK, SEK1, and MEKK1. JLP acts as a scaffold protein to bring together Max and c-Myc along with SAPK/JNK and p38, as well as their upstream SEK1 and MEKK3. MEKK1 itself plays a role as a scaffold protein that regulates SEK1 (Fig. 2C) (16). A theoretical consideration indicates that a single set of the scaffold-supported complex, which contains either SEK1 or MKK7, can not induce the sequential phosphorylation, since the synergistic activation of SAPK/JNK requires both SEK1 and MKK7 as observed in ES cells. Therefore, two sets of scaffold complexes, one containing SEK1 and the other MKK7, must exist closely in ES cells. Indeed, we have screened and found a predicted "binder"-like protein, which connects the two MAPKK (MKK7 and SEK1) cascades (Fig. 2D). Characterization of the protein in SAPK/JNK-signaling pathway is currently under investigation in our laboratory.

SAPK/JNK activation as a molecular switch in all-or-none manner

Recently, Ferrell et al have proposed an interesting concept that SAPK/JNK-signaling 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 (17, 18). They have shown in *Xenopus* oocytes, HeLa cells, HEK293 cells, and Jurkat T cells that SAPK/JNK responds to physiological and pathological stimuli, such as progesterone and sorbitol, in an all-or-none manner. 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 appeared to be switch-like. Indeed, we have also observed a very steep concentration-dependent response in the activation of SAPK/JNK by hyper-osmolar stress, sorbitol, in murine ES cells (Fig. 3) (10). 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

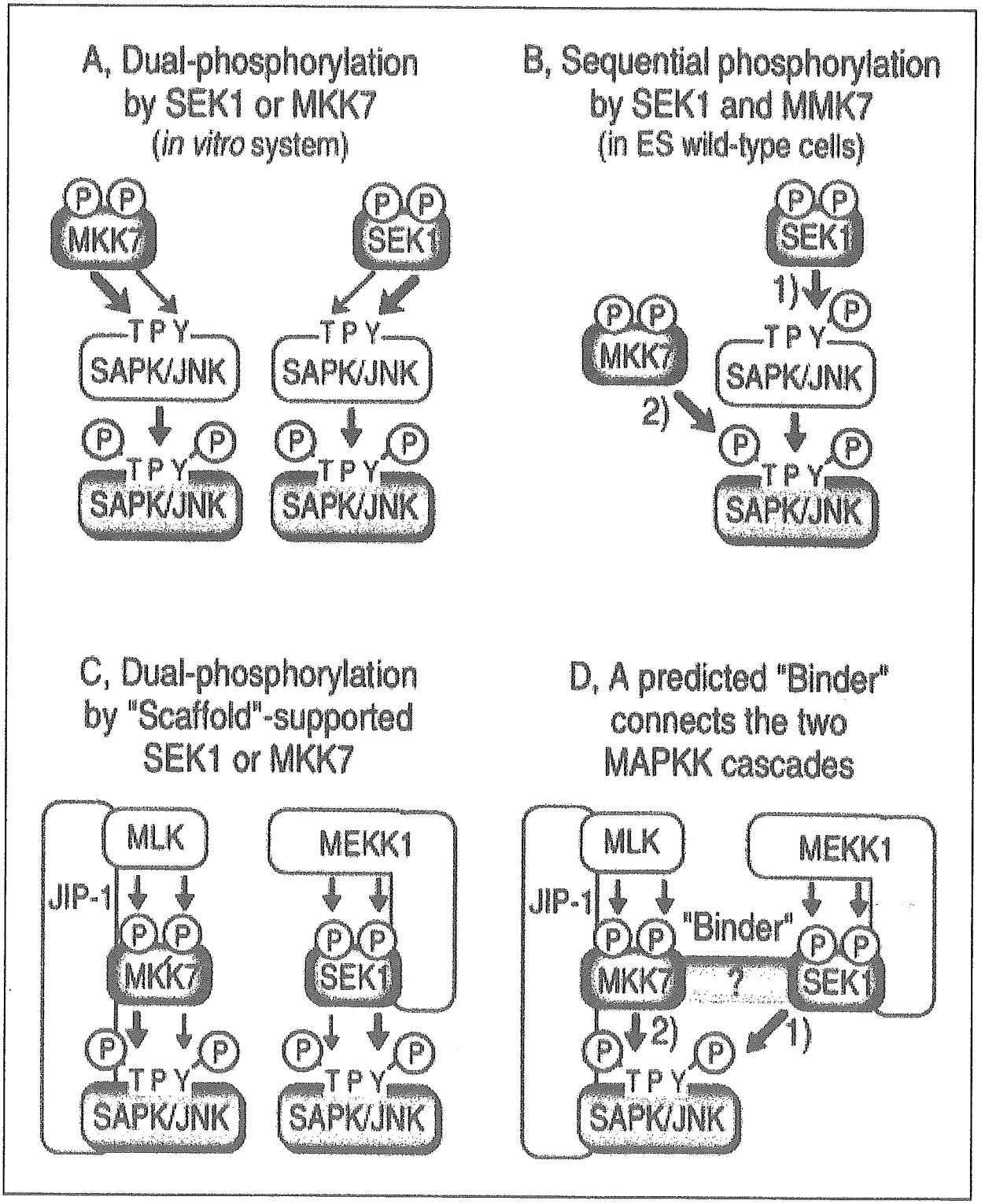

Fig. 2 - Schematic description of SAPK/JNK phosphorylation by SEK1 and MKK7 under various conditions. A, synergistic activation of SAPK/JNK by the dual-specificity kinase, SEK1 or MKK7, which has been reported in *In vitro* conditions (6-8). B, synergistic activation of SAPK/JNK through sequential phosphorylation by SEK1 and MKK7 in murine ES cells (9, 10). C, activation of SAPK/JNK by SEK1 or MKK7 associated with their scaffold proteins, JIP-1 and MEKK1 (11, 16). D, a predicted "binder" connects the two scaffold-supported MAPKK cascades (SEK1 and MKK7) for the sequential phosphorylation of SAPK/JNK as shown in panel B. TPY, Thr-Pro-Tyr motif.

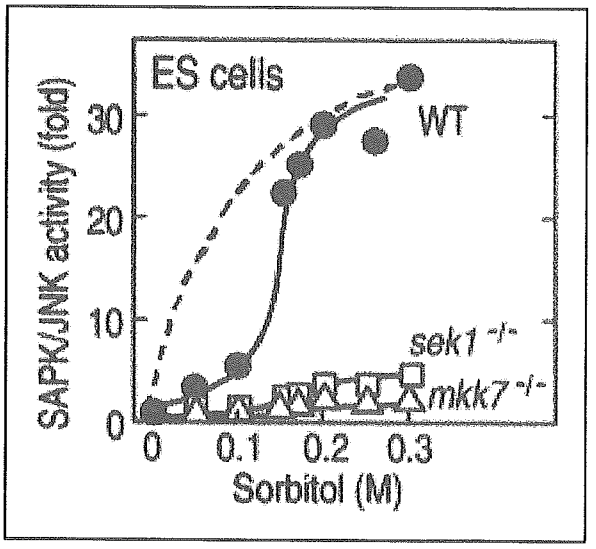

Fig. 3 - SAPK/JNK activation in response to hyper-osmolar stress (sorbitol) requires both SEK1 and MKK7 in ES cells. Wild-type, sek1$^{-/-}$, and mkk7$^{-/-}$ ES cells were stimulated with the indicated concentrations of sorbitol for 30 min.

proceed without errors basically through the two separated signals, one activating SEK1 and another activating 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 two separated pathways leading to SAPK/JNK activation may exist and physiologically function as a fail-safe mechanism as proposed previously (9).

Role of SAPK/JNK in mouse development

Sek1, mkk7, and all three Jnk (Jnk1, 2, and 3) loci have been knocked out at present. Sek1$^{-/-}$ embryos die between embryonic day 10.5 (E10.5) and E12.5 with impaired liver formation and massive apoptosis (Fig. 4) (19-21). We have recently shown that SEK1-mediated SAPK/JNK pathway downstream TNF-α receptor 1 (TNFR1) participates in embryonic hepatoblast proliferation and survival via a pathway different from NF-κB-induced anti-apoptosis. On the other hand, mkk7$^{-/-}$ embryos die between E11.5–12.5 with similar defects in liver formation. These results indicate that SAPK/JNK activation mediated through SEK1 plus MKK7 plays indispensable roles in hepatoblast proliferation and survival during mouse embryogenesis (Fig. 5) (22).

JNK1 and JNK2 are widely expressed in many tissues, but JNK3 is expressed predominantly in nervous system. Mice deficient in the single gene of Jnk1, Jnk2, or Jnk3, and Jnk1/Jnk3- or Jnk2/Jnk3-double mutant mice all survived normally. Mice lacking both JNK1 and JNK2 die around E11 with severe dysregulation of apoptosis in brain. Specifically, there was a reduction of cell death in the lateral edges of hindbrain prior to neural tube closure. In contrast, increased apoptosis and caspase activation were found in the mutant forebrain. These results assign both pro-and anti-apoptotic functions to JNK1 and JNK2 in the development of the fetal brain (23, 24).

Role of SAPK/JNK in cell survival and apoptosis

In mammalian cells, apoptotic signaling cascades can be divided into two broad categories: the intrinsic (mitochondria-dependent) and the extrinsic (death receptor-mediated) pathways. The initiation of mitochondria-dependent pathway requires a change in the organella membrane permeability that is prevented by anti-apoptotic molecules such as Bcl-2 and Bcl-X_L and promoted by pro-apoptotic molecules including Bax and Bak. The permeability change results in the release of mitochondrial proteins. One of the released proteins, cytochrome c, associates with Apaf1 and caspase 9 to activate the effector caspase 3 (25, 26). Cellular stresses such as UV irradiation and heat shock mediate apoptosis through the mitochondria-dependent pathway (27). However, upstream signaling that regulates the pro-apoptotic molecules remains to be elucidated. Recently, the involvement of SAPK/JNK activation in pro-apoptotic function has been suggested in the study with Jnk1$^{-/-}$ Jnk2$^{-/-}$ and mkk4$^{-/-}$ mkk7$^{-/-}$ mouse embryonic fibroblasts (MEFs). Both Jnk1$^{-/-}$ Jnk2$^{-/-}$ and mkk4$^{-/-}$mkk7$^{-/-}$ MEFs exhibited profound defects in stress-induced apoptosis (28, 29). Furthermore, it has been reported that JNK activation causes the release of apoptogenic factors such as cytochrome c and Smac from isolated mitochondria in a cell-free system (30, 31). These results strongly suggest that the SAPK/JNK activation directly regulates mitochondria-dependent apoptosis in pro-apoptotic direction.

Recently, we generated ES cells lacking both MKK4 and MKK7 to reevaluate the role of SAPK/JNK activation in the stress-induced and mitochondria-dependent apoptosis. We utilize mouse ES cells in terms of the following advantages: 1) ES cells are a prototype of all cell lineages and can be differentiated into MEF-like cells with retinoic acid, 2) ES cells do not express death receptors including Fas and TNFR1, but have stress-induced, mitochondria-dependent apoptotic pathway, and 3) the molecular mechanism of SAPK/JNK activation is well characterized in ES cells. Interestingly, SAPK/JNK activation is not required for stress-induced and mitochondria-dependent apoptosis in ES and MEF-like cells (submitted). Thus, the physiological role of SAPK/JNK activation in cell survival and apoptosis is still controversial, suggesting have an anti-apoptotic, a pro-apoptotic, or no function in these processes dependent on the types of cells and stimuli (32).

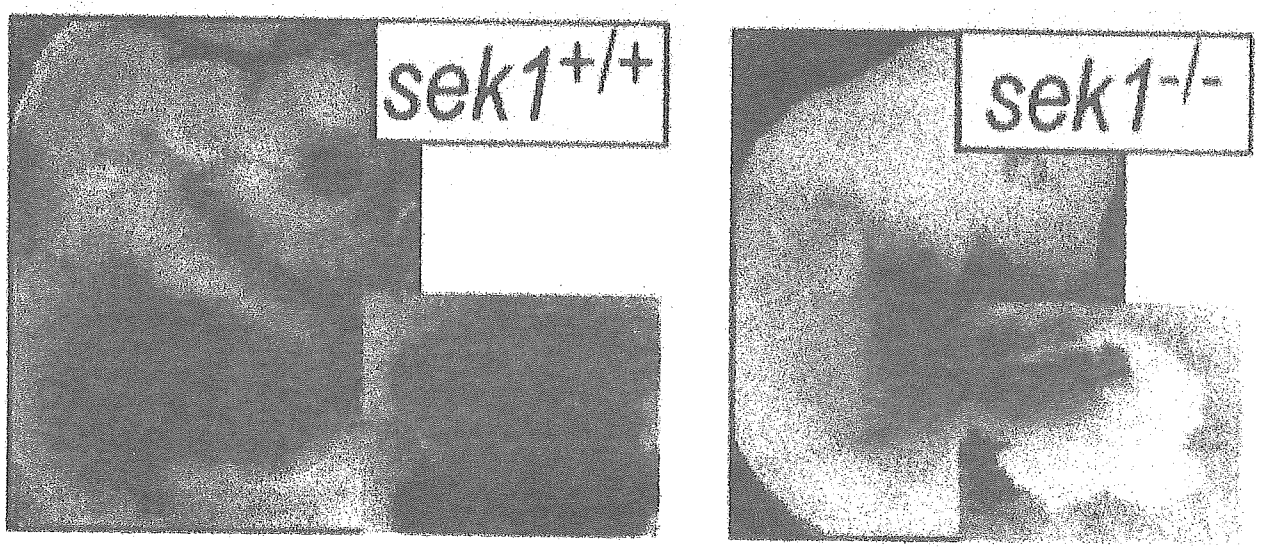

Fig. 4 - Defective liver formation in sek1$^{-/-}$ embryos. Appearance of wild-type and sek1$^{-/-}$ embryos and livers at embryonic day 12.5. Severe anemia is observed in sek1$^{-/-}$ embryos.

Role of SAPK/JNK in mouse immune responses

CD4^{+} and C8^{+} T cells are two subsets of peripheral T cells that play important roles during an immune response. After antigen stimulation, CD4^{+} T cells differentiate into effector Th1 or Th2 cells that secret cytokines to help modulate the type of immune response that is generated. Th1 cell promote cell-mediated immunity against intracellular microbial pathogens by expressing interferon-γ, interleukin (IL)-2, and lymphotoxin, whereas Th2 cells promote humoral immunity against parasites and extracellular pathgens by expressing IL-4, IL-5, IL-9, IL-10, and IL-13. CD8^{+} T cells differentiate into cytotoxic T cells to help defend the host during the cell-mediated immune response. Thus, defective T cell development to Th1 and Th2 cells results in impaired immune responses. Furthermore, inappropriate activation of T cells initiates and perpetuates many autoimmune diseases including rheumatoid, asthma, inflammatory bowel disease, and multiple sclerosis. It has been reported that T cells from mice deficient in the Jnk1 or Jnk2 gene have a defect in functional differentiation into Th1 or Th2 subsets (33, 34). Sabapathy et al have reported that JNK1 positively regulates mature T cell activation and that JNK1 and JNK2 have similar and overlapping roles in T cell function (35). Conze et al have also reported that JNK1 is required for CD8^{+} T cell activation, however, that JNK1 and JNK2 have distinct functions in CD4^{+} T cell differentiation and CD8^{+} T cell activation (36). Furthermore, we found defective T cell activation, whereas Swat et al. found normal activation of T cells lacking SEK1 (37, 38). These results indicate that the magnitude of the stimuli, the expression of JNK isoforms, and the activation level of SAPK/JNK are important factors determining the direction and efficiency of T cell activation (35). Thus, SAPK/JNK appears to be involved in the differentiation and activation of T cells, though its precise molecular mechanisms are still controversial.

Other physiological roles and target molecules of SAPK/JNK

As described above, SAPK/JNK regulates embryonic development including cell proliferation, survival, and apoptosis, and immune responses including T cell differentiation and activation. Furthermore, it has been reported that SAPK/JNK regulates mRNA stabilization, cell migration, and cytoskeletal integrity (Fig. 1). Turnover of mRNA is an important mechanism for the regulation of gene expression in organisms from bacteria to mammals. Regulation of mRNA half-life can influence normal cell proliferation, differentiation and oncogenesis. PB-3c mast cells produce IL-3 upon stimulation with extracellular signals, and its short-lived (half-life is about 30 min) mRNA is stabilized by Ca^{2+} ionophores. Using an active MEKK1 and a dominant-negative mutant of JNK, it has been indicated that SAPK/JNK is involved in the regulation of IL-3 mRNA turnover in mast cells (39). SAPK/JNK is required for *Drosophila* dorsal closure and is also essential for cell migration in mammalian cells. Rat bladder tumor epithelial cells (NBT-II) exhibit rapid keratinocyte-like movement. Interestingly, SP600125, a specific inhibitor of JNK, suppresses the movement. Several experiments

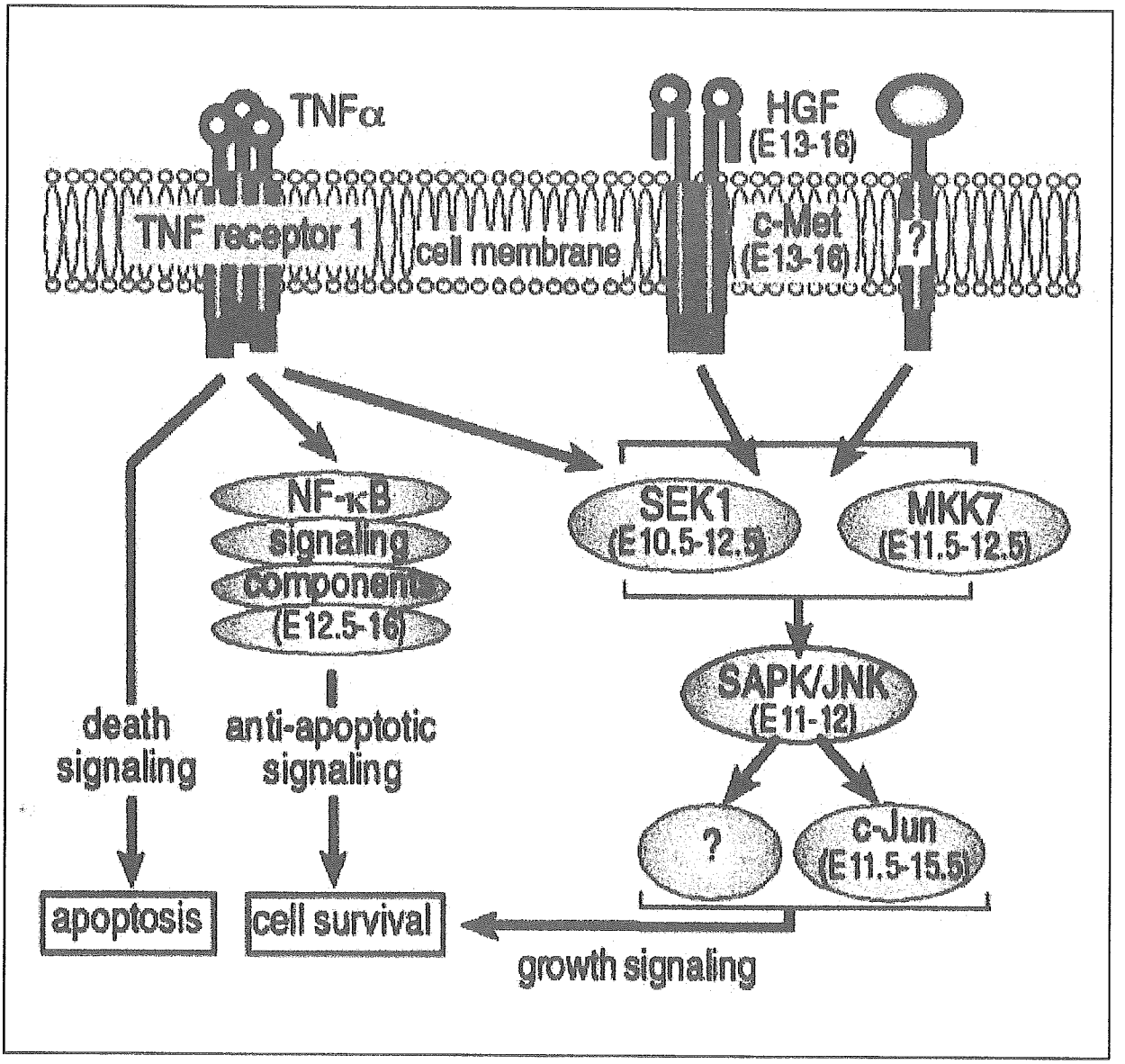

Fig. 5 - A proposed model for SAPK/JNK signaling pathway in hepatoblasts. The numbers in parentheses are dates of embryonic lethality reported in previous papers. TNFα elicits a wide range of biological responses, such as inflammation, tumor necrosis, differentiation, cell proliferation, and apoptosis, through the stimulation of its receptor, TNFR1. The induction of apoptosis, NF-κB activation, and SAPK/JNK activation are simultaneously mediated through TNFR1. SAPK/JNK activation is involved in cell growth, while activation of NF-κB protects against the apoptosis in hepatoblasts (22).

indicate that JNK1 phosphorylates serine 178 on paxillin, a focal adhesion adaptor, in NBT-II cells. Expression of a paxillin mutant (Ser178 to Ala) inhibited the migration of the cells. Thus, phosphorylation of paxillin by SAPK/JNK seems to be essential for maintaining the labile adhesions required for rapid cell migration (40). Dynamic assembly and disassembly of microtubules is essential for a variety of cellular functions, such as maintenance of cell morphology and polarity, cell division, cell locomotion, and intracellular trafficking. JNK1-deficient mice exhibit progressive degeneration of long nerve fibers and loss of microtubule integrity in dendrites. Dendritic degeneration of neuronal microtubules is associated with hypo-phosphorylation of microtubule assembly-promoting protein (MAP) 2 and its reduced ability to promote tubulin polymerization. Thus, JNK1 is required for maintaining the cytoskeletal integrity of neuronal cells and is a critical regulator of MAP activity and microtubule assembly (41).

Conclusions

Data continues to emerge implicating the SAPK/JNK-signaling pathway in a number of physiological functions that may be involved in human disease including autoimmune, anti-inflammatory, neurogenerative diseases, and cancers. In fact, mutations in Jnk3 gene were identified in human brain tumors (42). Similarly, mutations in sek1 gene have been identified in human cancers of pancreas, lung, breast, colon, and prostate as tumor suppressor genes (43-45). Under these circumstances, several pharmaceutical companies have been working on the discovery of SAPK/JNK-related drugs such as an anthrapyrazolone and SP600125. Efforts by many researchers in this field may help to find effective drugs in the near future.

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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 NH_2-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.

The SAPK/JNK[1] is a member of the family of mitogen-activated protein kinase (MAPK). This MAPK is activated not only 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-1β 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).

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