THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 3, Issue of January 16, pp. 1621–1626, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

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

Received for publication, September 17, 2003, and in revised form, October 24, 2003
Published, JBC Papers in Press, October 29, 2003, DOI 10.1074/jbc.M310335200

Gen Nishitai‡, Nao Shimizu‡, Takahiro Negishi‡, Hiroyuki Kishimoto‡, Kentaro Nakagawa‡, Daiju Kitagawa‡, Tomomi Watanabe‡, Haruka Momose‡, Shinya Ohata‡, Shuhei Tanemura‡, Satoshi Asaka‡, Junko Kubota‡, Ryota Saito‡, Hiroki Yoshida§, Tak W. Mak¶, Teiji Wada¶, Josef M. Penninger¶, Noriyuki Azuma‖, Hiroshi Nishina‡, and Toshiaki Katada‡**

From the ‡Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan, the §Department of Biomolecular Sciences, Saga Medical School, Saga 849-8501, Japan, the ¶University Health Network, Departments of Medical Biophysics and Immunology, the University of Toronto, Toronto, Ontario M5G 2C1, Canada, and the ‖Department of Ophthalmology, National Center for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan

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).

* This work was supported in part by research grants from the Ministry of Education, Science, Sports, and Culture and the Ministry of Health, Labor and Welfare of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: 81-3-5841-4754; Fax: 81-3-5841-4751; E-mail: nishina@mol.f.u-tokyo.ac.jp.

[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.

This is an Open Access article under the CC BY license.

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 $\times$ *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 $\times$ *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 $\times$ *g* for 10 min at 4 °C. Mitochondrial pellets were washed in 100 μl of buffer A followed by centrifugation at 10,000 $\times$ *g* for 10 min at 4 °C and lysed in 1.5$\times$ 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-

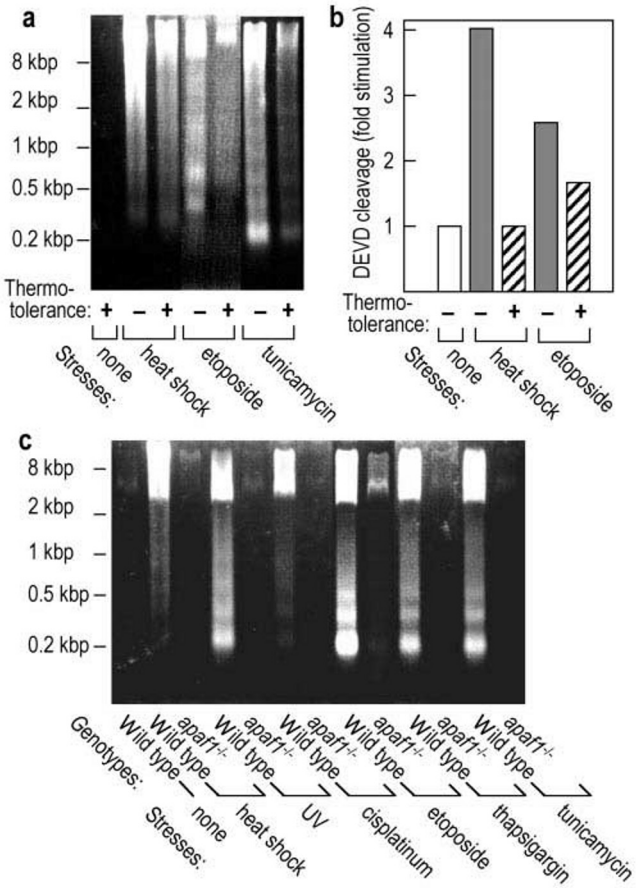

FIG. 1. **Characterization of stress-induced apoptosis in ES cells.** Wild-type ES cells were treated with (+) or without (−) heat shock at 44 °C for 10 min (*thermotolerance*), incubated for 6 h, and stimulated with the indicated stresses for 12 h. *a*, agarose-gel electrophoresis of DNA fragmentation. *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.

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.

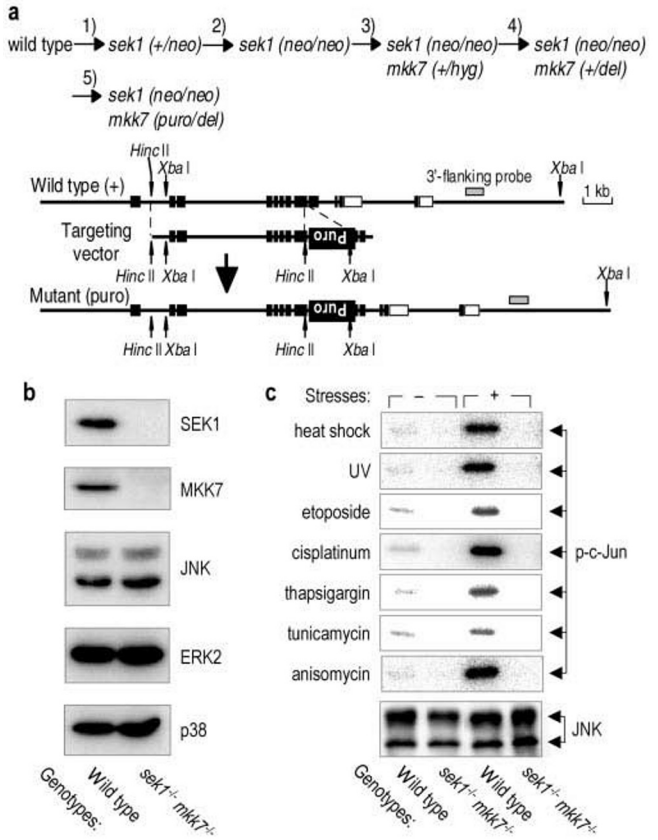

FIG. 2. **Targeted gene disruption of both *sek1* and *mkk7* in mouse ES cells.** *a*, flow chart of the disruption of *sek1* and *mkk7* genes and a novel *mkk7* (puromycin)-targeting vector. The genomic *mkk7* 3′-flanking probe used for Southern blotting is indicated as *gray boxes*, and the predicted 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.

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*$^{-/-}$

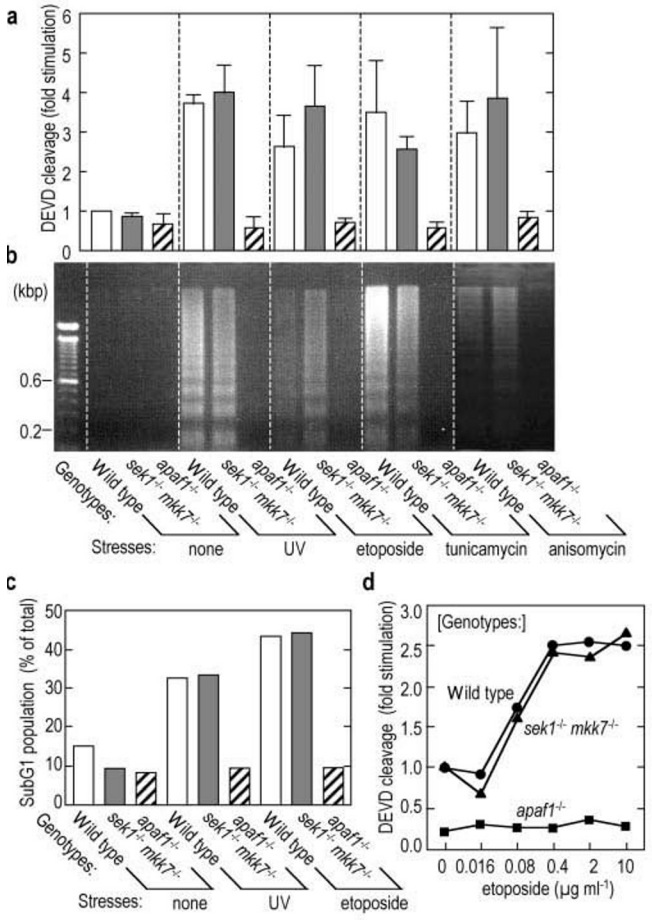

FIG. 3. **Stress-induced apoptosis in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ ES cells.** ES cells were stimulated with UV (20 J/m^2), etoposide (2 μg/ml), tunicamycin (10 μg/ml), or anisomycin (5 μg/ml) and further incubated for 12 h, and caspase 3 activation (*a*), DNA fragmentation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

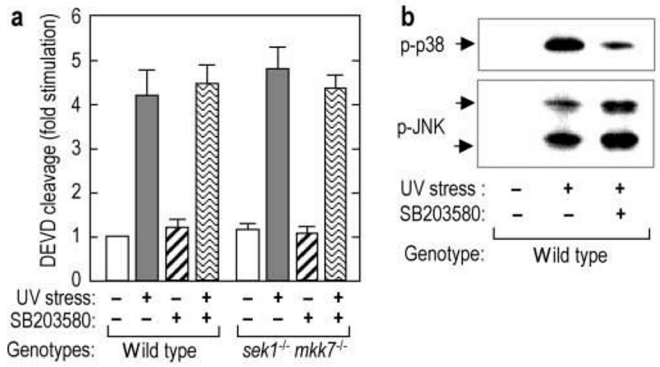

FIG. 4. **Effect of p38 inhibition on stress-induced apoptosis in ES cells.** *a*, ES cells were incubated with (+) or without (−) 10 μM SB203580 for 1 h, stimulated with UV (20 J/m^2), and further incubated for 12 h. UV-induced apoptosis was estimated by caspase 3 activation. *b*, ES cells were stimulated 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.

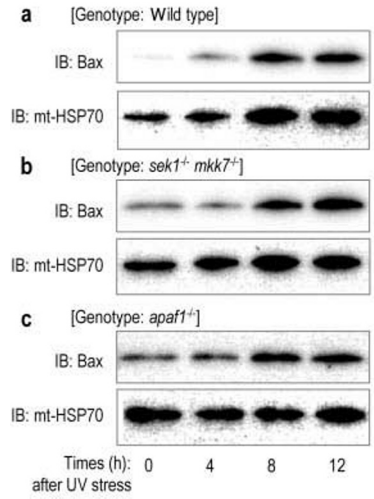

FIG. 5. **Stress-induced Bax translocation in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ ES cells.** ES cells were stimulated with UV (200 J/m^2) and further incubated for 4, 8, and 12 h. Mitochondrial membranes were prepared and immunoblotted (*IB*) with anti-Bax and anti-mitochondrial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

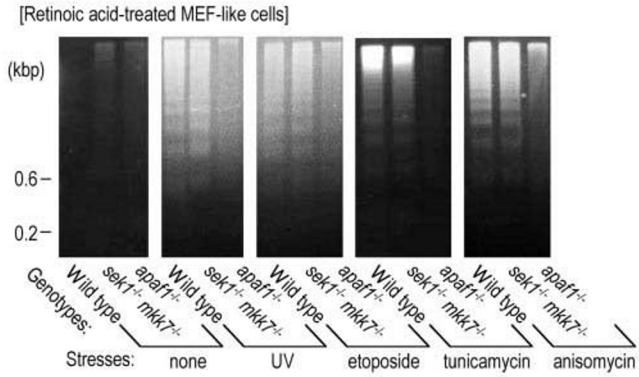

FIG. 6. **Stress-induced apoptosis in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, and *apaf1*$^{-/-}$ MEF-like cells.** ES cells were treated with 10 μM retinoic acid for 14 days and differentiated into MEF-like cells. The cells were stimulated UV (200 J/m^2), etoposide (20 μg/ml), tunicamycin (50 μg/ml),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

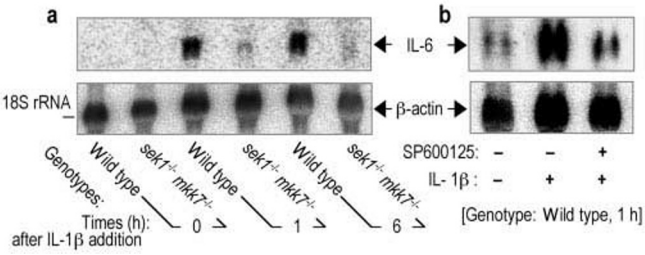

FIG. 7. **IL-1-induced IL-6 gene expression is impaired in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells.** *a*, wild-type and *sek1*$^{-/-}$ *mkk7*$^{-/-}$ MEF-like cells were stimulated with 100 ng/ml IL-1β for 1 and 6 h. *b*, wild-type MEF-like cells were incubated with (+) or without (−) 50 μM SP600125 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 *sek*1$^{-/-}$ 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.

Acknowledgments—We thank Dr. Izumu Saito and RIKEN BRC for providing us with adenovirus-encoding Cre recombinase. We also thank Dr. James R. Woodgett for suggestions and criticism.

REFERENCES

1. Peng, L., Deepak, N., Imawati, B., Srinivasa, M. S., Manzoor A., Emad, S. A., and Xiaodong, W. (1997) *Cell* **91,** 479–489
2. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) *Nature Rev. Mol. Cell Biol.* **4,** 552–565
3. Yoshida, H., Kong, Y.-Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998) *Cell* **94,** 739–750
4. Davis, R., J. (2000) *Cell* **103,** 239–252
5. Chang, L., and Karin M. (2001) *Nature* **410,** 37–40
6. Manning, A. M., and Davis, R. J. (2003) *Nature Rev. Mol. Drug Disc.* **2,** 554–565
7. Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998) *Curr. Biol.* **8,** 1387–1390
8. Wada, T., Nakagawa, K., Watanabe, T., Nishitai, G., Seo, J., Kishimoto, H., Kitagawa, D., Sasaki, T., Penninger, J. M., Nishina, H., and Katada, T. (2001) *J. Biol. Chem.* **276,** 30892–30897
9. Kishimoto, H., Nakagawa, K., Watanabe, T., Kitagawa, D., Momose, H., Seo, J., Nishitai, G., Shimizu, N., Ohata, S., Tanemura, S., Asaka, S., Goto, T., Fukushi, H., Yoshida, H., Suzuki, A., Sasaki, T., Wada, T., Penninger, J. M., Nishina, H., and Katada, T. (2003) *J. Biol. Chem.* **278,** 16595–16601
10. Ganiatsas, S., Kwee, L., Fujiwara, Y., Perkins, A., Ikeda, T., Labow, M. A., and Zon, L. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6881–6886
11. Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., Pompa, J. L., Furlonger, K., Paige, C., Hui, C.-C., Fischer, K. D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J. R., and Penninger, J. M. (1999) *Development* **126,** 505–516
12. Watanabe, T., Nakagawa, K., Ohata, S., Kitagawa, D., Nishitai, G., Seo, J., Tanemura, S., Shimizu, N., Kishimoto, H., Wada, T., Aoki, J., Arai, H., Iwatsubo, T., Mochita, M., Watanabe, T., Satake, M., Ito, Y., Matsuyama, T., Mak, T. W., Penninger, J. M., Nishina, H., and Katada, T. (2002) *Dev. Biol.* **250,** 332–347
13. Lin, A. (2002) *BioEssays* **25,** 17–24
14. Kuan, C.-Y., Yang, D. D., Roy, D. R. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) *Neuron* **22,** 667–676
15. Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M., and Wagner, E. F. (1999) *Mech. Dev.* **89,** 115–124
16. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
17. Tournier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., and Davis, R. J. (2001) *Genes Dev.* **15,** 1419–1426
18. Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M., and Izumo, S. (2002) *J. Biol. Chem.* **277,** 10244–10250
19. Chauhan, D., Li, G., Hideshima, T., Podar, K., Mitsiades, C., Mitsiades, N., Munshi, N., Kharbanda, S., and Anderson, K. C. (2003) *J. Biol. Chem.* **278,** 17593–17596
20. Nishina, H., Fischer, K. D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E. A., Bernstein, A., Mak, T. W., Woodgett, J. R., and Penninger, J. M. (1997) *Nature* **385,** 350–353
21. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sasaki, T., Sugano, S., and Saito, I. (1995) *Nucleic Acids Res.* **23,** 3816–3821
22. Kitagawa, D., Tanemura, S., Ohata, S., Shimizu, N., Seo, J., Nishitai, G., Watanabe, T., Nakagawa, K., Kishimoto, H., Wada, T., Tezuka, T., Yamamoto, T., Nishina, H., and Katada, T. (2002) *J. Biol. Chem.* **277,** 366–371
23. Nishina, H., Bachmann, M., Oliveira-dos-Santos, A. J., Kozieradzki, I., Fischer, K. D., Odermatt, B., Wakeham, A., Shahinian, A., Takimoto, H., Bernstein, A., Mak, T. W., Woodgett, J. R., Ohashi, P. S., and Penninger, J. M. (1997) *J. Exp. Med.* **186,** 941–953
24. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) *Nat. Cell Biol.* **2,** 469–475
25. Saleh, A., Srinivasula, S. M., Balkir, L., Robbins, P. D., and Alnemri, E. S. (2000) *Nat. Cell Biol.* **2,** 476–483
26. Bruey, J.-M., Ducasse, C., Bonniaud, P., Ravagnant, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A.-P., Kroemer, G., Solary, E., and Garrido, C. (2000) *Nat. Cell Biol.* **2,** 645–652
27. Xia, Z. G., Dickens, M., Raigeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1331
28. Putcha, G. V., Le, S., Frank, S., Besirli, C. G., Clark, K., Chu, B., Alix, S., Youle, R. J., LaMarche, A., Maroney, A. C., and Johnson, E. M., Jr. (2003) *Neuron* **38,** 899–914
29. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Benneriah, Y. (1994) *Cell* **77,** 727–736
30. Conze, D., Krahl, T., Kennedy, N., Weiss, L., Lumsden, J., Hess, P., Flavell, R. A., Gros, G. L., Davis, R. J., and Rincon M. (2002) *J. Exp. Med.* **195,** 811–823
31. Krause, A., Holtmann, H., Eickemeier, S., Winzen, R., Szamel, M., Resch, K., Saklatvala, J., and Kracht, M. (1998) *J. Biol. Chem.* **273,** 23681–23689
32. Holtmann, H., Enninga, J., Kalble, S., Thiefes, A., Dorrie, A., Broemer, M., Winzen, R., Wilhelm, A., Ninomiya-Tsuji, J., Matsumoto, K., Resch, K., and Kracht, M. (2001) *J. Biol. Chem.* **276,** 3508–3516