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Differential Regulation of HSP70 Expression by the JNK Kinases SEK1 and MKK7 in Mouse Embryonic Stem Cells Treated With Cadmium

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Abstract JNK, a member of the mitogen-activated protein kinases (MAPKs), is activated by the MAPK kinases SEK1 and MKK7 in response to environmental stresses. In the present study, the effects of $CdCl_2$ treatment on MAPK phosphorylation and HSP70 expression were examined in mouse embryonic stem (ES) cells lacking the *sek1* gene, the *mkk7* gene, or both. Following $CdCl_2$ exposure, the phosphorylation of JNK, p38, and ERK was suppressed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ cells. When *sek1*$^{-/-}$ or *mkk7*$^{-/-}$ cells were treated with $CdCl_2$, JNK phosphorylation, but not the phosphorylation of either p38 or ERK, was markedly reduced, while a weak reduction in p38 phosphorylation was observed in *sek1*$^{-/-}$ cells. Thus, both SEK1 and MKK7 are required for JNK phosphorylation, whereas their role in p38 and ERK phosphorylation could overlap with that of another kinase. We also observed that $CdCl_2$-induced HSP70 expression was abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ cells, was reduced in *sek1*$^{-/-}$ cells, and was enhanced in *mkk7*$^{-/-}$ cells. Similarly, the phosphorylation of heat shock factor 1 (HSF1) was decreased in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and *sek1*$^{-/-}$ cells, but was increased in *mkk7*$^{-/-}$ cells. Transfection with siRNA specific for JNK1, JNK2, p38α, ERK1, or ERK2 suppressed $CdCl_2$-induced HSP70 expression. In contrast, silencing of p38β or p38γ resulted in further accumulation of HSP70 protein. These results suggest that HSP70 expression is up-regulated by SEK1 and down-regulated by MKK7 through distinct MAPK isoforms in mouse ES cells treated with $CdCl_2$. J. Cell. Biochem. 104: 1771–1780, 2008.

Key words: MAPK; SEK1; MKK7; HSP70; embryonic stem cells

Mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr kinases that are activated by mitogens or stress conditions and play an essential role in a diverse array of cellular functions such as proliferation, differentiation, development, migration, inflammatory responses, and apoptosis [Schaeffer and Weber, 1999; Chang and Karin, 2001; Kyriakis and Avruch, 2001; Raman and Cobb, 2003; Qi and Elion, 2005; Raman et al., 2007]. Upon activation, MAPKs phosphorylate and control the activity of key cytoplasmic molecules and nuclear proteins, which in turn regulate expression of specific genes. There are three primary families of MAPKs in mammalian systems, including extracellular signal-regulated protein kinases (ERK1 and ERK2), c-Jun NH_2-terminal kinases (JNK1, JNK2, and JNK3), also known as stress-activated protein kinases (SAPKγ, SAPKα, and SAPKβ), and p38 MAPKs (p38α, p38β, p38γ, and p38δ). ERK3, ERK5, and ERK7 are additional MAPKs with distinct regulatory roles [Raman et al., 2007].

Activation of MAPKs requires the dual phosphorylation at a Thr-X-Tyr motif (where X is Glu in ERK, Pro in JNK, and Gly in p38) in the activation loop of the kinase domain subdomain VIII by MAPK kinases (MAPKK, MAP2K,

Abbreviations used: $CdCl_2$, cadmium chloride; ERK, extracellular signal-regulated kinase; ES, embryonic stem; HSF1, heat shock factor 1; HSP70, heat shock protein 70; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK and MKK, MAPK kinase; MEK, MAPK/ERK kinase; RT-PCR, reverse-transcription polymerase chain reaction; SEK, stress-activated protein kinase/ERK kinase; siRNA, short interfering RNA; WT, wild type.

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MEK, MKK, or SEK) [Kyriakis and Avruch, 2001]. These MAPK kinases are specific for individual MAPK members, where ERK1/2 are activated by MEK1 and MEK2, JNK is activated by SEK1 (also known as MKK4 or MEK4) and MKK7 (also known as SEK2), p38 is activated by MKK3 and MKK6, and ERK5 is activated by MEK5 [Kyriakis and Avruch, 2001]. This specificity is not universal as SEK1 contributes to the activation of p38 in vitro [Dérijard et al., 1995; Doza et al., 1995; Lin et al., 1995] and in vivo when overexpressed [Wang et al., 1997; Guan et al., 1998; Hickson et al., 2006], and is physiologically required for p38 activation in mouse embryonic fibroblasts treated with ultraviolet irradiation [Brancho et al., 2003]. Furthermore, mouse T cells targeting the *mkk7* gene have been shown to exhibit much higher p38 activity than wild-type T cells [Dong et al., 2000]. These findings suggest that the JNK kinases SEK1 and MKK7 regulate both JNK and p38, both of which are signaling pathways activated in response to various environmental stresses [Kyriakis and Avruch, 2001]. The possible regulation of ERK by JNK kinases is yet unknown.

To clarify the involvement of SEK1 and MKK7 in the activation of MAPK proteins, we examined the phosphorylation status of JNK, p38, and ERK in mouse embryonic stem (ES) cells lacking the *sek1* gene, the *mkk7* gene, or both (double knockout) following treatment with cadmium chloride ($CdCl_2$), a toxic metal compound that induces the marked phosphorylation of all three MAPK families in mouse ES cells [Matsuoka et al., 2004]. In addition, the expression of heat shock protein 70 (HSP70), one of the stress response proteins regulated by MAPKs through the phosphorylation of heat shock factor 1 (HSF1) [Hung et al., 1998; Park and Liu, 2001], was examined in this system. Next, MAPK modules responsible for $CdCl_2$-induced HSP70 expression were determined using short interfering RNAs (siRNAs) specific for MAPK proteins JNK1, JNK2, p38α, p38β, p38γ, p38δ, ERK1, and ERK2. These results suggest that SEK1 and MKK7 might regulate the phosphorylation of JNK, p38, and ERK with different redundancy in mouse ES cells treated with $CdCl_2$. Furthermore, we suggest the possibility that $CdCl_2$-induced HSP70 expression is differentially regulated by JNK kinases and downstream MAPK isoforms.

MATERIALS AND METHODS

Chemicals

$CdCl_2$ was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Polyclonal antibodies against phosphorylated SAPK/JNK (Thr183/Tyr185), total SAPK/JNK, phosphorylated p38 (Thr180/Tyr182), total p38, phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, and total MKK7 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin (I-19) polyclonal antibody, anti-MEK-4 (SEK1) (C-20) polyclonal antibody, and anti-JNK1 (FL) polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-HSP70 (C92F3A-5) monoclonal antibody and anti-HSF1 (10H8) monoclonal antibody were from Assay Designs, Inc. (Ann Arbor, MI).

Cell Culture and Treatments

sek1$^{-/-}$, *mkk7*$^{-/-}$, and double knockout *sek1*$^{-/-}$ *mkk7*$^{-/-}$ mutant cell lines were made by targeting the *sek1* gene, the *mkk7* gene, or both from the murine ES cell line E14K (wild type) as described [Nishina et al., 1997; Kishimoto et al., 2003; Nishitai et al., 2004]. All cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Invitrogen Corp., Carlsbad, CA) supplemented with 15% fetal calf serum and leukemia inhibitory factor as described previously [Nishina et al., 1997]. No definite difference in the cell growth between wild-type and mutated ES cells was found (data not shown). For each experiment, exponentially growing ES cells were plated at 5×10^5 cells/well in 6-well culture plates, 1×10^6 cells/60-mm culture dishes, or 7×10^5 cells/12.5-cm^2 culture flasks, and cultured overnight or for 24 h (for siRNA transfection) before the experiment was performed. $CdCl_2$ was dissolved in water and filter sterilized before use. Cells were incubated in serum-free medium containing 1 μM $CdCl_2$ for 3 or 6 h. In heat shock experiments, cells were incubated at 44°C for 10 min, and then at 37°C for 30 min, 3 or 6 h in serum-free medium.

siRNA Transfection

Duplexed stealth siRNA targeted against the mouse *sek1* [designed using the BLOCK-iT RNAi Designer (Invitrogen)], *mkk7* (Map 2k7-MSS218515), *jnk1* (Mapk8-MSS218562), *jnk2* (Mapk9-MSS218566), *p38α* (Mapk14-

MSS240942), *p38β* (Mapk11-MSS207969), *p38γ* (Mapk12-MSS248775), *p38δ* (Mapk13-MSS218556), *erk1* (Mapk3-MSS218560), and *erk2* (Mapk1-MSS218549) genes were synthesized by Invitrogen Corp. The 25-mer siRNA sequences were as follows: SEK1, 5′-UUCAGAAUUACUCAGCUGCGGAGGG-3′; MKK7, 5′-AAGGCAGUCUUUGACAAAUGACUGG-3′; JNK1, 5′-AUAAGCUGCACACACUAUUCCUUGA-3′; JNK2, 5′-UUCAAUCGCAUGCUCUCUUUCUUCC-3′; p38α, 5′-AUGUGCUUCAGCAGACGCAACUCUC-3′; p38β, 5′-UUCACCGCUACAUUGCUGGGCUUCA-3′; p38γ, 5′-AUAUUUCUGGGCCUUGGGUUCAUCC-3′; p38δ, 5′-AAUCAUGGAAGCUCCGAAGGGAAGA-3′; ERK1, 5′-AAUGUAAACAUCUCUCAUGGCUUCC-3′; and ERK2, 5′-AUAAUACUGCUCCAGGUAUGGGUGG-3′. Each siRNA or Stealth RNAi Negative Control (Invitrogen) was transfected into wild-type ES cells at the time of plating using Lipofectamine 2000 (Invitrogen) following instructions from the manufacturer.

Western Immunoblotting

At harvest, cells were washed with phosphate-buffered saline (PBS) and lysed in Laemmli sample buffer. Cell lysates were sonicated and boiled for 5 min. Protein concentration was determined using the RC DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein [30 or 10 μg (Fig. 2A)] were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk or bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The membrane was incubated overnight at 4°C with primary antibody, and protein was detected using a Phototope-HRP Western blot detection kit (Cell Signaling Technology) or a SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Chemical Co., Rockford, IL). Some blots were incubated with Restore Western Blot Stripping Buffer (Pierce) for 30 min at room temperature and reprobed with anti-actin, anti-total JNK, anti-total p38, or anti-total ERK antibody.

RT-PCR

Reverse-transcription polymerase chain reaction (RT-PCR) analysis for the semiquantification of mRNA was carried out as described previously [Liu et al., 2006]. The cDNA fragments corresponding to mouse HSP70.1, β-actin, JNK3, p38β, p38γ, and p38δ were amplified using the following primers: HSP70.1, 5′-AGGAGGCCGAGCGCTACAAG-3′ and 5′-TACAGTGCACAGTGCTGCTC-3′; β-actin, 5′-CATCACTATTGGCAACGAGC-3′ and 5′-ACGCAGCTCAGTAACAGTCC-3′; JNK3, 5′-CACGTGGATGTGTCATCTATTGC-3′ and 5′-TGCTCGATGACTTTGTTCCACTG-3′ [Xu et al., 2003]; p38β, 5′-CCTGGCACCCATGAAATTGAGC-3′ and 5′-TGCCTGAGAGGCGCCTCTTG-3′; p38γ, 5′-GCCAGAGTTCCAAAGGAGACG-3′ and 5′-GAAGCCAAGGTGCCGTGAAG-3′; and p38δ, 5′-GCGACGAAGTGGCATGAAGC-3′ and 5′-GCTCCAGGGCTACACAGTAAG-3′.

All of the experiments were repeated a minimum of three times.

RESULTS

$CdCl_2$-Induced Phosphorylation of MAPKs Is Suppressed in ES Cells Lacking Both SEK1 and MKK7

To define the involvement of SEK1 and MKK7 in the activation of MAPKs, phosphorylated forms of JNK, p38, and ERK were assessed in wild-type, $sek1^{-/-}$ $mkk7^{-/-}$ double knockout, and $sek1^{-/-}$ or $mkk7^{-/-}$ single knockout ES cells following exposure to 1 μM $CdCl_2$ for 3 or 6 h (Fig. 1). At the end of these exposures, definite cellular damages were not found with a WST-8 conversion assay (data not shown). In wild-type ES cells, each phosphorylated MAPK increased in intensity after 3 or 6 h, whereas the amount of total MAPKs were not changed during this incubation period. In $sek1^{-/-}$ $mkk7^{-/-}$ ES cells, $CdCl_2$-induced JNK phosphorylation was completely abolished. Furthermore, $CdCl_2$-induced phosphorylation of p38 and ERK was markedly suppressed in $sek1^{-/-}$ $mkk7^{-/-}$ ES cells. Suppression of the phosphorylation of JNK, p38, and ERK was also observed in $sek1^{-/-}$ $mkk7^{-/-}$ ES cells treated with a higher dose (10 μM) of $CdCl_2$ (data not shown). Although the possibility that the signaling pathways leading to p38 or ERK activation in $sek1^{-/-}$ $mkk7^{-/-}$ ES cells are altered cannot be excluded, these findings suggest that SEK1 and MKK7 are required for phosphorylation of not only JNK, but also p38 and ERK in mouse ES cells treated with $CdCl_2$. When $sek1^{-/-}$ or $mkk7^{-/-}$ single knockout ES cells were treated with 1 μM $CdCl_2$ for 3 or 6 h, p38 and ERK phosphorylation was not reduced

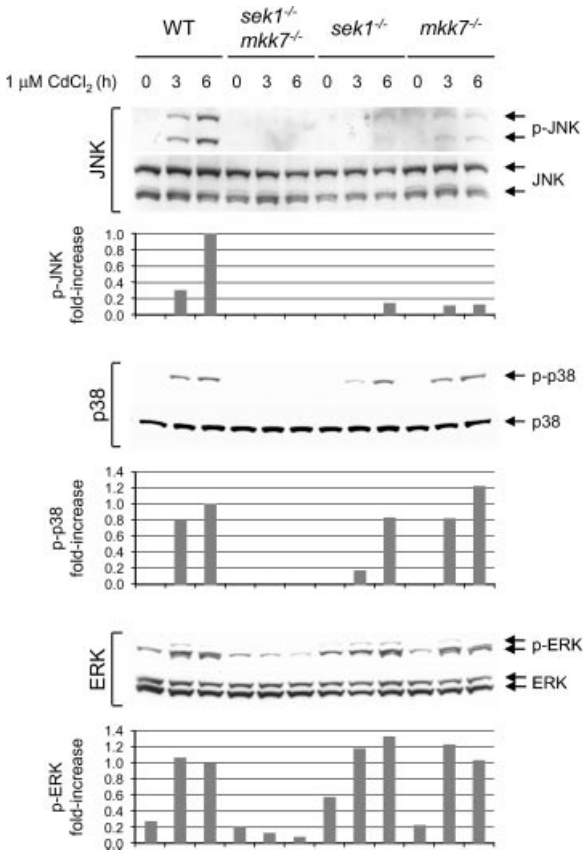

Fig. 1. Effects of treatment with $CdCl_2$ on the amount of phosphorylated JNK, p38, and ERK in wild-type (WT), *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells. Cells were either untreated (0 h) or treated with 1 μM $CdCl_2$ for 3 or 6 h. Cell lysates were subjected to Western immunoblot using anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK, and anti-ERK antibodies. In the densitometric analysis, each value was expressed as the ratio of phosphorylated MAPK level to the corresponding total MAPK level, and the value of WT cells treated with $CdCl_2$ for 6 h was set to 1. Results shown are representative of three independent experiments.

to significant levels, but a weak reduction of p38 phosphorylation was observed in *sek1*$^{-/-}$ ES cells. These results indicate that SEK1 and MKK7 have a nonredundant role in the phosphorylation of JNK, but a redundant role in the phosphorylation of p38 and ERK in mouse ES cells treated with $CdCl_2$.

$CdCl_2$-Induced Accumulation of HSP70 and Phosphorylation of HSF1 Are Suppressed in ES Cells Lacking Both SEK1 and MKK7

To further examine the physiological role of SEK1 and MKK7, expression of HSP70, a stress response protein up-regulated by cadmium treatment [Beyersmann and Hechtenberg, 1997], was assessed by Western immunoblotting (Fig. 2A) and RT-PCR analysis (Fig. 2B) in wild-type, *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells. In wild-type ES cells treated with $CdCl_2$, the levels of HSP70 mRNA and protein increased after 3 and 6 h, respectively (Fig. 2A,B). $CdCl_2$-induced HSP70 expression was completely abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells, and reduced significantly in *sek1*$^{-/-}$ ES cells. In contrast, the amount of both HSP70 protein and its mRNA were elevated in *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$. It has been shown that the phosphorylation of HSF1 is required for HSP70 expression by cadmium treatment [Holmberg et al., 2001; Pirkkala et al., 2001]. In wild-type ES cells, treatment with $CdCl_2$ resulted in the accumulation of phosphorylated HSF1, which was detected by an increase in size on the immunoblots (Fig. 2A). Similarly, phosphorylation-dependent band-shift of HSF1 was

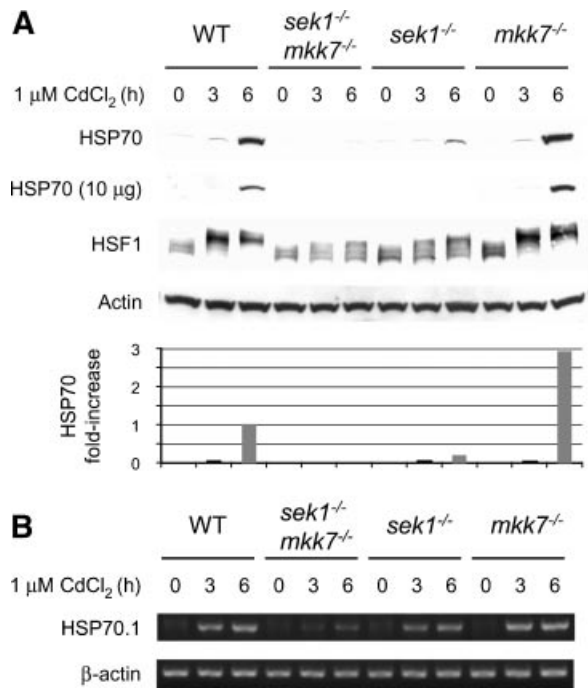

Fig. 2. Effects of treatment with $CdCl_2$ on the expression of HSP70 in wild-type (WT), *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells. Cells were either untreated (0 h) or treated with 1 μM $CdCl_2$ for 3 or 6 h. **A**: Cell lysates were subjected to Western immunoblot using anti-HSP70, anti-HSF1, and anti-actin antibodies. Ten micrograms of lysates other than 30 μg were applied for the HSP70 immunoblot to show the definite elevation of HSP70 protein level in *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$. In the densitometric analysis, each value was expressed as the ratio of HSP70 level to actin level, and the value of WT cells treated with $CdCl_2$ for 6 h was set to 1. **B**: Total RNA was isolated and subjected to RT-PCR analysis using HSP70.1 and β-actin primers. Results shown are representative of three independent experiments.

suppressed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and *sek1*$^{-/-}$ ES cells, but was enhanced in *mkk7*$^{-/-}$ ES cells. Transfection of siRNA targeted against the *mkk7* gene into *sek1*$^{-/-}$ ES cells and the *sek1* gene into *mkk7*$^{-/-}$ ES cells reduced the expression of MKK7 and SEK1, respectively (Fig. 3A). In both cases, $CdCl_2$-induced HSP70 expression was significantly suppressed when compared with *sek1*$^{-/-}$ or *mkk7*$^{-/-}$ ES cells transfected with negative control siRNA (Fig. 3B). Collectively, these findings suggest that both SEK1 and MKK7 are required at least for the initial induction of HSP70 expression following exposure to $CdCl_2$, whereas they have opposite effects on the amplification of HSP70 expression.

Signaling Pathways Leading to HSP70 Expression Are Different Between Heat Shock and $CdCl_2$ Treatment

We next tested if heat shock treatment could induce expression of HSP70 in the presence of absence of SEK1 or MKK7 similar to that in mouse ES cells treated with $CdCl_2$. When compared with $CdCl_2$, weak phosphorylation of JNK, p38, and ERK was observed in heat shocked wild-type ES cells (Fig. 4A). JNK phosphorylation disappeared completely in mutated (*sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$) ES cells. On the other hand, the phosphorylation of p38 and ERK was retained substantially, indicating that SEK1 and MKK7 are not critical for phosphorylation of p38 and ERK in heat shocked mouse ES cells. In wild-type ES cells, the levels of HSP70 protein and its corresponding mRNA increased at 3 h post-heat shock (Fig. 4B,C). Unlike $CdCl_2$ treatment, heat shock-induced HSP70 expression was not completely abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells, and was not suppressed in *sek1*$^{-/-}$ ES cells. However, HSP70 accumulated to high levels in heat shocked *mkk7*$^{-/-}$ ES cells. Thus, it is possible that JNK kinases are not a major pathway responsible for HSP70 expression in heat shocked mouse ES cells.

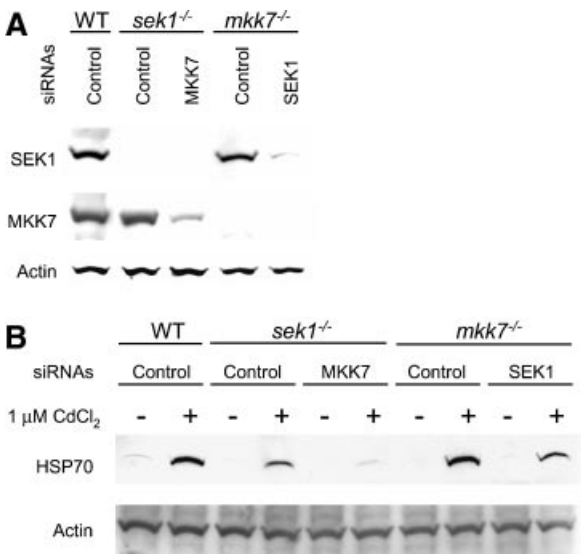

Fig. 3. Effects of knockdown of MKK7 in *sek1*$^{-/-}$ ES cells and of SEK1 in *mkk7*$^{-/-}$ ES cells on $CdCl_2$-induced HSP70 expression. Wild-type (WT), *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells were transfected with siRNA negative control (control) or siRNA specific for the *mkk7* gene (for *sek1*$^{-/-}$ ES cells) or the *sek1* gene (for *mkk7*$^{-/-}$ ES cells). **A**: Cell lysates were subjected to Western immunoblot using anti-SEK1 and anti-MKK7 antibodies. **B**: Cells were either untreated (−) or treated (+) with 1 μM $CdCl_2$ for 6 h. Cell lysates were subjected to Western immunoblot using anti-HSP70 and anti-actin antibodies. Results shown are representative of three independent experiments.

MAPK Isoforms Contribute Differentially to $CdCl_2$-Induced HSP70 Expression

To investigate the role of MAPKs in this system, siRNAs targeted against the MAPK isoforms JNK1, JNK2, p38α, p38β, p38γ, p38δ, ERK1, and ERK2 were transfected into wild-type ES cells (Fig. 5). Because the *jnk3* gene is primarily expressed in brain, heart, and testis [Davis, 2000], and the expression of JNK3 in wild-type ES cells (E14K) was barely detectable by immunoblot and RT-PCR analysis (data not shown), we did not employ siRNA targeted against JNK3. The expression of JNK1, JNK2, p38α, ERK1, and ERK2 was specifically reduced in immunoblots using available antibodies against each protein. Since anti-p38 antibody used in this study detected dominantly p38α (Fig. 5B), the silencing of the β, γ, and δ p38 isoforms was confirmed by RT-PCR analysis (data not shown). The knockdown of JNK1, JNK2, p38α, ERK1, and ERK2 in wild-type ES cells resulted in the marked suppression of $CdCl_2$-induced accumulation of HSP70 protein (Fig. 5A–C). In contrast, silencing of p38β and p38γ enhanced $CdCl_2$-induced HSP70 expression (Fig. 5B), and p38δ silencing did not affect HSP70 expression. Thus, among siRNAs targeted against MAPK isoforms, p38β and p38γ siRNAs could enhance $CdCl_2$-induced HSP70 expression in wild-type ES cells as was observed in *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$ (Fig. 2). To examine the relationship between JNK kinases and p38 isoforms, siRNA targeted against p38α, p38β, p38γ, or p38δ was

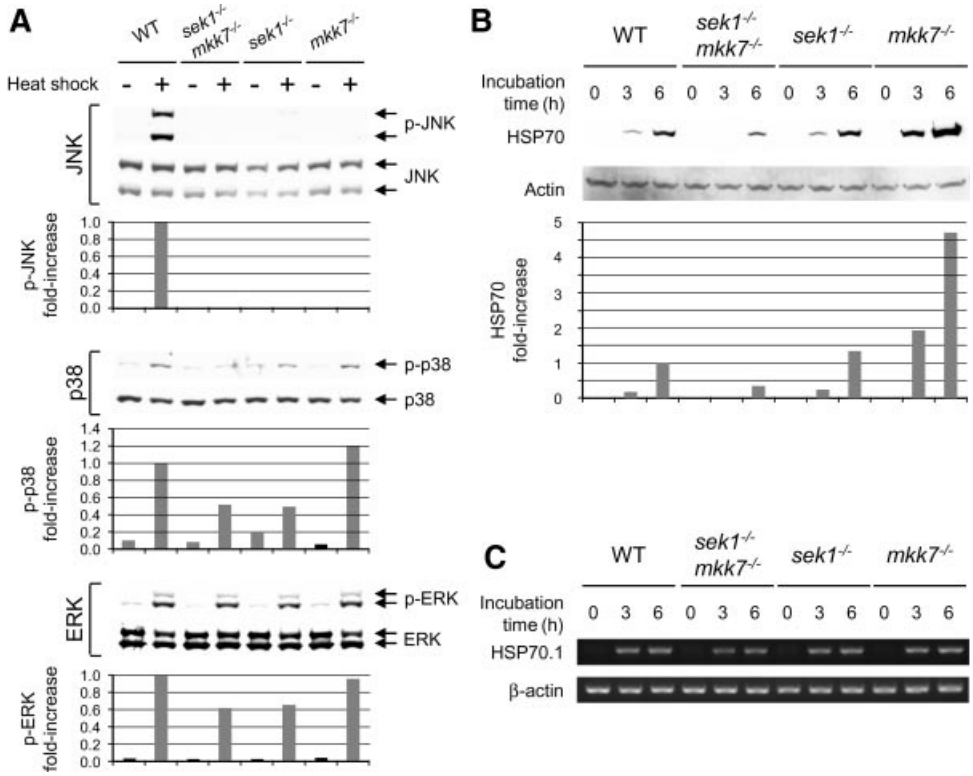

Fig. 4. Effects of heat shock treatment on the amount of phosphorylated MAPKs (**A**), HSP70 protein (**B**), and its mRNA (**C**) in wild-type (WT), *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells. A: Cells were either untreated (−) or stimulated (+) with a pulse of heat shock at 44°C for 10 min and further incubated at 37°C for 30 min. Cell lysates were subjected to Western immunoblot using anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK, and anti-ERK antibodies. In the densitometric analysis, each value was expressed as the ratio of phosphorylated MAPK level to the corresponding total MAPK level, and the value of WT cells treated with heat shock was set to 1. B,C: Cells were either untreated (0 h) or stimulated with a pulse of heat shock at 44°C for 10 min and further incubated at 37°C for 3 or 6 h. Cell lysates were subjected to Western immunoblot using anti-HSP70 and anti-actin antibodies. In the densitometric analysis, each value was expressed as the ratio of HSP70 level to actin level, and the value of WT cells treated with heat shock (6 h) was set to 1 (B). Total RNA was isolated and subjected to RT-PCR analysis using HSP70.1 and β-actin primers (C). Results shown are representative of three independent experiments.

transfected into *sek1*$^{-/-}$ and *mkk7*$^{-/-}$ ES cells (Fig. 5D). Suppression of $CdCl_2$-induced accumulation of HSP70 protein in *sek1*$^{-/-}$ ES cells was restored by the transfection of siRNA targeted against p38β or p38γ. Elevation of $CdCl_2$-induced HSP70 expression in *mkk7*$^{-/-}$ ES cells was not further amplified by silencing p38β or p38γ. These results indicate that p38β and p38γ could be activated by MKK7 to repress HSP70 expression. On the other hand, silencing of p38α in *mkk7*$^{-/-}$ ES cells resulted in the marked suppression of $CdCl_2$-induced HSP70 expression, while that in *sek1*$^{-/-}$ ES cells had no effect. It is possible that in contrast to β and γ isoforms, p38α might be activated by SEK1 to promote HSP70 expression in mouse ES cells treated with $CdCl_2$.

DISCUSSION

In the present study, we examined the roles of SEK1 and MKK7 in MAPKs phosphorylation and HSP70 expression using single or double knockout mutants of these JNK kinases. The accumulation of phosphorylated forms of JNK, p38, and ERK was observed simultaneously when wild-type ES cells were treated with 1 μM $CdCl_2$ (Fig. 1). In *sek1*$^{-/-}$ *mkk7*$^{-/-}$ double knockout ES cells, $CdCl_2$-induced phosphorylation of these three MAPK families was significantly suppressed. However, when *sek1*$^{-/-}$ or *mkk7*$^{-/-}$ single knockout ES cells were treated with 1 μM $CdCl_2$, the phosphorylation of JNK but not p38 and ERK was significantly suppressed, while a weak reduction in p38

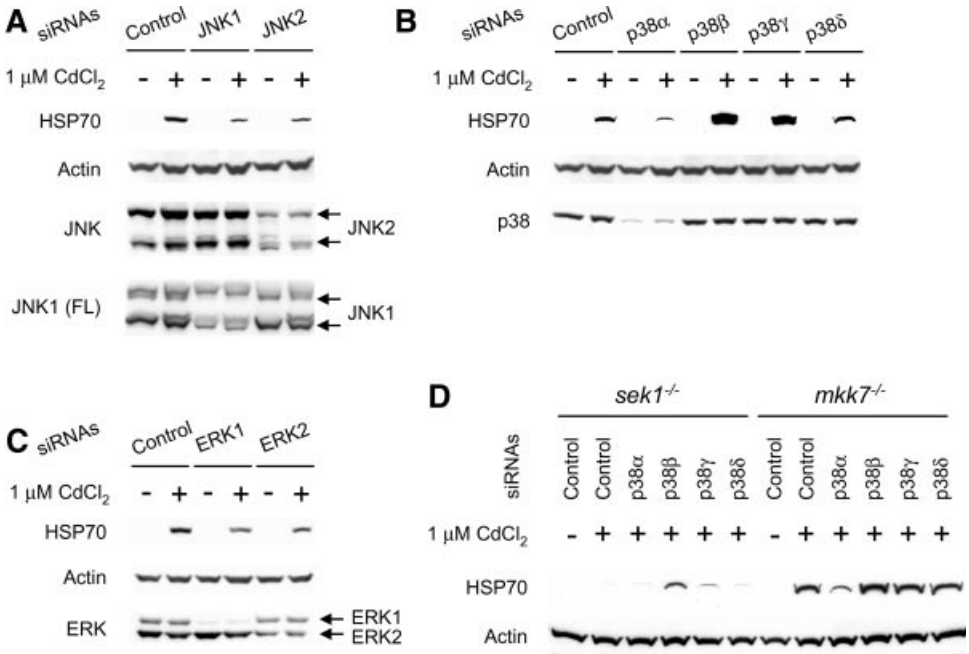

Fig. 5. Effects of MAPK isoform knockdown on $CdCl_2$-induced HSP70 expression. Wild-type ES cells were transfected with siRNA negative control (control) or siRNA specific for the *jnk1* and *jnk2* genes (**A**), the *p38α*, *p38β*, *p38γ*, and *p38δ* genes (**B**), or the *erk1* and *erk2* genes (**C**), and then untreated (−) or treated (+) with 1 μM $CdCl_2$ for 6 h. Cell lysates were subjected to Western immunoblot using anti-HSP70 and anti-actin antibodies (A–C), anti-JNK (JNK2) and anti-JNK1 (FL) (JNK1/p46) antibodies (A), anti-p38 antibody (B), and anti-ERK antibody (C). **D**: *sek1*$^{-/-}$ and *mkk7*$^{-/-}$ ES cells were transfected with siRNA negative control (control) or siRNA specific for the *p38α*, *p38β*, *p38γ*, and *p38δ* genes, and then untreated (−) or treated (+) with 1 μM $CdCl_2$ for 6 h. Cell lysates were subjected to Western immunoblot using anti-HSP70 and anti-actin antibodies. Results shown are representative of three independent experiments.

phosphorylation was observed in *sek1*$^{-/-}$ ES cells. These findings suggest that SEK1 and MKK7 might contribute to the phosphorylation of MAPK members with different redundancy. The phosphorylation of JNK on Tyr and Thr residues is preferentially induced by SEK1 and MKK7, respectively [Weston and Davis, 2002]. It has been reported that the activation of JNK by ultraviolet irradiation, heat shock, sorbitol, anisomycin, cadmium, and inorganic mercury is attenuated in mouse ES cells targeting either the *sek1* or the *mkk7* gene [Wada et al., 2001; Kishimoto et al., 2003; Matsuoka et al., 2004]. This study employed *sek1*$^{-/-}$ *mkk7*$^{-/-}$ double knockout ES cells treated with $CdCl_2$ [and heat shock (Fig. 4A)] and confirmed that both SEK1 and MKK7 are required for JNK phosphorylation. In contrast, the role of SEK1 or MKK7 in the phosphorylation of p38 and ERK appears to be redundant with another JNK kinase. While MKK3 and MKK6 are immediately upstream of p38 [Kyriakis and Avruch, 2001], in vitro studies suggest that SEK1 can activate p38 [Dérijard et al., 1995; Doza et al., 1995; Lin et al., 1995]. Co-transfection of 293 cells with SEK1 and p38 resulted in the activation of p38 [Wang et al., 1997]. In cultured cells, overexpression of a constitutively active form of SEK1 activated both p38 and JNK, whereas that of the inactive form did not [Guan et al., 1998; Hickson et al., 2006]. Furthermore, the decreased expression of SEK1 by siRNA suppressed p38 activation in *mkk3*$^{-/-}$ *mkk6*$^{-/-}$ double knockout mouse embryonic fibroblasts treated with ultraviolet irradiation [Brancho et al., 2003]. In the present study, the phosphorylation of p38 in *sek1*$^{-/-}$ ES cells was less significant than in wild-type ES cells following exposure to $CdCl_2$ (Fig. 1). Collectively, the data indicate that SEK1 retains the ability to activate both JNK and p38 in response to environmental stresses including cadmium. The possible involvement of MKK7 in p38 phosphorylation, and of SEK1 and MKK7 in ERK phosphorylation, have not been previously observed, probably because their roles are redundant with each other. Since heat shock-induced phosphorylation of p38 and ERK was not

completely abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ double knockout ES cells (Fig. 4A), it is possible that JNK kinases regulate MAPK modules depending on the nature of the stimuli. It remains to be determined whether these JNK kinases (SEK1 and MKK7) can phosphorylate p38 and ERK molecules, or modulate their upstream kinases (MKK3/6 for p38, and MEK1/2 for ERK) in mouse ES cells treated with $CdCl_2$.

We also examined the expression of HSP70, a stress response protein induced by cadmium [Beyersmann and Hechtenberg, 1997], in *sek1*$^{-/-}$ *mkk7*$^{-/-}$, *sek1*$^{-/-}$, and *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$. $CdCl_2$-induced HSP70 expression was abolished in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells, and was significantly reduced in *sek1*$^{-/-}$ ES cells (Fig. 2). Unexpectedly, the expression of HSP70 was enhanced in *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$. Consistent with the finding that the transcriptional activity of HSF1, which binds to the heat shock element located within the *hsp70* gene promoter [Morimoto, 1998], requires phosphorylation on Ser230 [Holmberg et al., 2001], the phosphorylation of HSF1 decreased in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and *sek1*$^{-/-}$ ES cells, and increased in *mkk7*$^{-/-}$ ES cells following exposure to $CdCl_2$ (Fig. 2A). These findings suggest that SEK1 might activate, whereas MKK7 might suppress, the transcriptional activation of the *hsp70* gene through the regulation of HSF1 phosphorylation. Both SEK1 and MKK7 are likely required for the initial induction of HSP70 expression, because $CdCl_2$-induced HSP70 expression was suppressed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ double knockout ES cells (Fig. 2), *sek1*$^{-/-}$ ES cells transfected with siRNA targeting MKK7, and *mkk7*$^{-/-}$ ES cells transfected with siRNA targeting SEK1 (Fig. 3B). In contrast to $CdCl_2$, heat shock-induced HSP70 expression was still observed in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells (Fig. 4B,C). Taken together with the findings that $CdCl_2$, but not heat shock, induced the phosphorylation of p38 and ERK as well as JNK depending on the presence of SEK1 and MKK7 (Figs. 1 and 4A), it is possible that JNK kinase-MAPK cascades regulate the expression of HSP70 in mouse ES cells.

It has been reported that the activation of ERK by 60 μM $CdCl_2$ or that of p38 by 100 μM $CdCl_2$ transactivates the *hsp70* gene through the phosphorylation of HSF1 in 9L rat brain tumor cells [Hung et al., 1998]. Likewise, JNK, ERK, and p38 have been reported to phosphorylate the transcriptional activation domain of HSF1 in HeLa cells [Park and Liu, 2001]. We previously observed that LL-Z1640-2, an inhibitor of JNK and p38 pathways, suppressed HSP70 expression in NIH3T3 cells treated with 10 μM $CdCl_2$ [Sugisawa et al., 2004]. Consistent with these findings, $CdCl_2$-induced HSP70 expression was suppressed when expression of JNK1, JNK2, p38α, ERK1, or ERK2 was reduced by transfection with siRNA (Fig. 5A–C). In contrast, the silencing of p38β or p38γ resulted in enhancement of $CdCl_2$-induced HSP70 expression. Thus, $CdCl_2$-induced HSP70 expression appears to be differentially regulated by respective MAPK isoforms. The lack of JNK phosphorylation was accompanied with the suppression of HSP70 expression in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ and *sek1*$^{-/-}$ ES cells treated with $CdCl_2$ (Figs. 1 and 2). In *mkk7*$^{-/-}$ ES cells, however, $CdCl_2$-induced HSP70 expression was enhanced despite the absence of JNK phosphorylation. These findings suggest that JNK1 and JNK2 might play a role in the initial induction but not the amplification of HSP70 expression in response to $CdCl_2$ exposure. It remains to be determined whether ERK1, ERK2, and p38α are also responsible for the initial induction or the amplification of HSP70 expression.

Accumulating evidence indicates that p38 isoforms have distinct roles in the cellular functions such as activating proteins 1 (AP-1)-mediated transcription, proliferation, and apoptosis [Nemoto et al., 1998; Wang et al., 1998; Pramanik et al., 2003; Tanos et al., 2005; Askari et al., 2007]. The substrate specificity derived from carboxyl-terminal sequences of p38 isoforms might underlie these differences [Sabio et al., 2005]. The present experiments using siRNAs revealed that p38α promotes, while p38β and p38γ repress HSP70 expression in mouse wild-type ES cells treated with $CdCl_2$ (Fig. 5B). Together with the observation that $CdCl_2$-induced HSP70 expression was suppressed in *sek1*$^{-/-}$ ES cells and enhanced in *mkk7*$^{-/-}$ ES cells (Fig. 2), it is possible that p38α might be activated by SEK1 to promote HSP70 expression, and p38β and p38γ might be activated by MKK7 to repress HSP70 expression in response to $CdCl_2$ exposure. Consistent with this hypothesis, silencing of p38α reduced HSP70 expression in *mkk7*$^{-/-}$ ES cells, but did not affect it in *sek1*$^{-/-}$ ES cells. Conversely, the silencing of p38β or p38γ restored HSP70 expression in *sek1*$^{-/-}$ ES cells, but did not affect its expression in *mkk7*$^{-/-}$ ES cells treated with $CdCl_2$ (Fig. 5D). Further investigations are

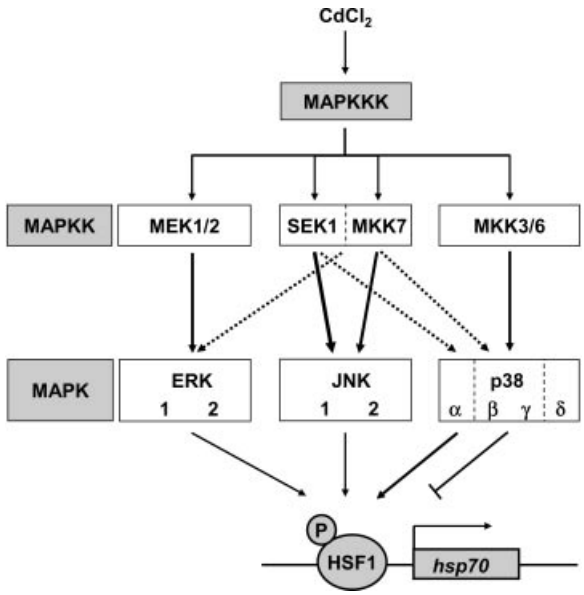

Fig. 6. The proposed role of SEK1 and MKK7 in HSP70 expression in mouse ES cells treated with $CdCl_2$. Following exposure to $CdCl_2$, SEK1 and MKK7 regulate the phosphorylation of ERK, JNK, and p38. The activation of ERK1, ERK2, JNK1, JNK2, and p38α by SEK1 promotes HSP70 expression through the phosphorylation of HSF1. The possible activation of p38β and p38γ by MKK7 represses HSP70 expression.

required to clarify the regulatory mechanisms of HSP70 expression by the isoforms of p38 and their upstream kinases.

In summary, the present study suggests that both SEK1 and MKK7 are required for the phosphorylation of JNK, and the role of either of these JNK kinases in the phosphorylation of ERK and p38 might be redundant with another kinase (Fig. 6). It is also possible that SEK1 and MKK7 either activate or suppress expression of HSP70, respectively, and that induced expression of HSP70 by $CdCl_2$ treatment is likely regulated in a complicated manner by JNK kinases and downstream MAPK isoforms.

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