Impaired Synergistic Activation of Stress-activated Protein Kinase SAPK/JNK in Mouse Embryonic Stem Cells Lacking SEK1/MKK4

DIFFERENT CONTRIBUTION OF SEK2/MKK7 ISOFORMS TO THE SYNERGISTIC ACTIVATION

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Stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), which is a member of the mitogen-activated protein kinase (MAPK) family, plays an important role in a stress-induced signaling cascade. SAPK/JNK activation requires the phosphorylation of Thr and Tyr residues in its Thr-Pro-Tyr motif, and SEK1 (M KK4) and MKK7 (SEK2) have been identified as the upstream MAPK kinases. Here we examined the activation and phosphorylation sites of SAPK/JNK and differentiated the contribution of SEK1 and MKK7α, -γ1, and -γ2 isoforms to the MAPK activation. In SEK1-deficient mouse embryonic stem cells, stress-induced SAPK/JNK activation was markedly impaired, and this defect was accompanied with a decreased level of the Tyr phosphorylation. Analysis in HeLa cells co-transfected with the two MAPK kinases revealed that the Thr and Tyr of SAPK/JNK were independently phosphorylated in response to heat shock by MKK7γ1 and SEK1, respectively. However, MKK7α1 failed to phosphorylate the Thr of SAPK/JNK unless its Tyr residue was phosphorylated by SEK1. In contrast, MKK7γ2 had the ability to phosphorylate both Thr and Tyr residues. In all cases, the dual phosphorylation of the Thr and Tyr residues was essentially required for the full activation of SAPK/JNK. These data provide the first evidence that synergistic activation of SAPK/JNK requires both phosphorylation at the Thr and Tyr residues in living cells and that the preference for the Thr and Tyr phosphorylation sites was different among the members of MAPK kinases.

The SAPK/JNK1 is a member of the family of mitogen-activated protein kinases (MAPKs). This MAPK is activated not only by many types of cellular stresses including heat shock, UV irradiation, and inflammatory cytokines (IL-1β and tumor necrosis factor-α) but also by heterotrimeric G-proteins, phorbol esters, and co-stimulatory activation of T lymphocytes. The activated SAPK/JNK phosphorylates c-Jun, Jun D, and ATF-2 to regulate gene expression for the stress response (1).

To examine the physiological roles of SAPK/JNK in the stress-induced signaling pathway, we previously disrupted sek1 gene in mouse embryonic stem (ES) cells and in mice (2, 3). In the sek1(−/−) ES cells, heat shock-induced activation of SAPK/JNK was almost completely abolished, indicating that SEK1 certainly functions as an activator of SAPK/JNK. However, evidence has indicated that there might be SAPK/JNK activator(s) other than SEK1. Indeed, several groups including us have isolated the cDNA encoding SEK2 (also called MKK7) as another activator of SAPK/JNK (4–10). There are six different isoforms of MKK7 (α1, α2, β1, β2, γ1, and γ2) due to alternative splicing from the same gene (11). SEK1-deficient embryos displayed severe anemia and died between embryonic day 10.5 and embryonic day 12.5 mainly because of defective liver formation (3, 12). However, biochemical roles of SEK1 in the liver formation in vivo remain to be resolved. Interestingly, SAPK/JNK activation in response to phorbol ester plus Ca²⁺ ionophore was lost in sek1(−/−) thymocytes but not in sek1(−/−) peripheral T cells (13). These findings allow us to speculate that SEK1-induced phosphorylation of SAPK/JNK may contribute to the kinase activation in different manners dependent on cell types.

In this regard, Lawler et al. (14), using Escherichia coli-expressed recombinant enzymes, have shown that SAPK1c/JNK1 was activated synergistically in vitro by SEK1 and MKK7 and that SEK1 had a preference for a Tyr residue and that MKK7 had a preference for a Thr residue within the Thr-Pro-Tyr motif in the MAPK. Lisnok et al. (15) also reported similar results using recombinant JNK3α1 expressed in E. coli. In a more detailed analysis with SAPK/JNK isoforms (JNK1α1, JNK2α2, and JNK3α1) expressed in insect cells, it has been shown that both SEK1 and MKK7 were required in vitro for the maximum activation (16). These results give us a hint why two kinds of SAPK/JNK activators, SEK1 and MKK7, exist in cells and explain the differences of the biochemical phenotypes in SEK1-deficient cells to some extent.

To elucidate the role of SEK1 in the SAPK/JNK activation in

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1 The abbreviations used are: SAPK/JNK, stress-activated protein kinase/c-Jun NH₂-terminal kinase; Ab, antibody; mAb, monoclonal Ab; ERK, extracellular signal-regulated kinase; ES, embryonic stem; GST, glutathione S-transferase; IL, interleukin; MAPK, mitogen-activated protein kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase kinase; MAPKK and MKK, MAPK kinase; HA, hemagglutinin; PIPes, 1,4-piperazinediethanesulfonic acid.

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Fig. 1. Time course of heat shock-induced stimulation of SAPK/JNK activity in wild-type and sek1(-/-) ES cells. Wild-type and sek1(-/-) ES cells were stimulated with a pulse of heat shock at 44 °C for 10 min (open box) and further incubated at 37 °C for the indicated times. Cell lysates were prepared from the cells and immunoprecipitated with an anti-SAPK/JNK (C-17) Ab. SAPK/JNK activity in the precipitated fractions was measured with GST-c-Jun as a substrate in the presence of [γ-32P]ATP as described under “Experimental Procedures.” The insets show the 32P-phosphorylated GST-c-Jun, and the activity is expressed as the -fold stimulation compared with the control level observed in sek1(-/-) ES cells without heat shock. The data shown are representative of three independent experiments.

Fig. 2. Activation of the MAPK family, ERK, p38, and SAPK/JNK, by UV irradiation in wild-type and sek1(-/-) ES cells. Wild-type and sek1(-/-) ES cells were stimulated with UV irradiation for 15 min. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting (IB) using anti-phospho-ERK (catalog no. 9105, panel A), anti-ERK (C-14 and C-16, panel B), anti-phospho-p38 (catalog no. 9211, panel C), anti-p38 (C-20, panel D), anti-phospho-SAPK/JNK (catalog no. 9251, panel E), and anti-SAPK/JNK (C-17, panel F) Abs. SEK1 and MKK7 proteins, after being immunoprecipitated (IP) with anti-SEK1 (KN-001) and anti-MKK7 (KN-004) mAbs, were also separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot with different anti-SEK1 (K-18) and anti-MKK7 (C-19) Abs (panels G and H) as described under “Experimental Procedures.” The data shown are representative of three independent experiments.

RESULTS

Impaired Stress-induced SAPK/JNK Activation in sek1-deficient ES Cells—To investigate the role of SEK1 in stress-induced SAPK/JNK activation in intact cells, we first used sek1(-/-) mouse ES cells that lacked SEK1 protein. Fig. 1 shows the time course of SAPK/JNK activity in response to a pulse of heat shock in wild-type and sek1(-/-) ES cells. The
activity was measured by the ability of SAPK/JNK to phosphorylate GST-c-Jun as a substrate. The heat shock markedly stimulated SAPK/JNK activity in wild-type ES cells; the maximum activation was observed at 30 min. However, such SAPK/JNK activation was greatly impaired in sek1Δ ES cells (Fig. 3, panels A and D). It has recently been reported that the phosphorylation of two amino acid residues on the Thr-Pro-Tyr motif within the T-loop of SAPK/JNK is essential for its full activation in the stimulated ES cells (11, 14–16). Therefore, we identified the phosphorylated sites of SAPK/JNK in the stimulated ES cells using anti-phospho-Tyr (PY20). The Tyr phosphorylation was almost completely abolished in the SEK1-deficient ES cells (Fig. 3, panels A and D). These results indicate that SEK1 is essential for the UV-induced full activation of SAPK/JNK and is not involved in the activation of other members of MAPK family, such as ERK and p38, in ES cells. In other words, the y1 isoform of MKK7 present in ES cells is not capable of compensating for the role of SEK1 in the stress-induced SAPK/JNK activation.

**Impaired Stress-induced SAPK/JNK Activation in SEK1-deficient ES Cells Arising from the Loss of Its Tyrosine Phosphorylation**—Besides heat shock and UV irradiation, a variety of stimuli, such as 1,10-phenanthroline, lysophosphatidic acid, and IL-1β, also induced SAPK/JNK activation to different extents in wild-type ES cells (Fig. 3). This activation was again markedly impaired in sek1Δ ES cells (Fig. 3, panels A and D). It has been recently reported that the phosphorylation of two amino acid residues on the Thr-Pro-Tyr motif within SAPK/JNK was required for the full activation of the MAPK in vitro (14–16). Therefore, we identified the phosphorylated sites of SAPK/JNK in the stimulated ES cells using anti-phospho-Tyr (PY20). The Tyr phosphorylation was almost completely abolished in the SEK1-deficient ES cells (Fig. 3, panels B and E). These results indicate that SEK1 preferentially phosphorylates the Tyr residue of the MAPK in ES cells. Thus, it is very likely that the SAPK/JNK full activation observed in wild-type ES cells seems to be because of the cooperative actions of SEK1 and MKK7 (y1) to phosphorylate both sites on the MAPK.

**Synergistic Activation of SAPK/JNK by Dual Phosphorylation of Its Thr and Tyr Residues**—In ES cells, SEK1 clearly contributed to the Tyr phosphorylation of SAPK/JNK, and the stress-induced full activation of the MAPK appeared to require the dual phosphorylation on the Thr-Pro-Tyr motif. Therefore, we further investigated the cooperative action of MKK7, which was supposed to be involved in the Thr phosphorylation of SAPK/JNK (14–16). For the analysis, we used HeLa cells for transient transfection because of low efficiency of transfection into ES cells. FLAG-tagged MKK7 (α1, γ1, or γ2) was co-expressed with FLAG-SEK1 and HA-JNK1/SAPKγ in the cells.
using pCMV5 mammalian vectors. The three isoforms of MKK7 used in this study were the shortest α1 (347 amino acids), γ1 which has an 89-residue extension at the amino terminus of α1, and the longest γ2, which has a 33-residue extension at the carboxyl terminus of γ1 resulting from alternative splicing of one gene locus (see Fig. 5A later). The transfected cells, after being starved for 12 h to decrease the basal activity of endogenous SAPK/JNK, were stimulated with heat shock at 42 °C for 15 min. The cell lysates were immunoprecipitated with anti-HA Affinity Matrix and analyzed for activity and phosphorylation of SAPK/JNK using anti-phospho Abs.

In a series of the experiments, the expression of HA-tagged JNK1 was almost constant (see Figs. 4–6, panel C). Endogenous SEK1 and MKK7 in HeLa cells did neither phosphorylate nor activate HA-tagged JNK1 under the present conditions (Figs. 4–6, lane 1). The different ratios of SEK1 and MKK7 expression vectors induced varied expression of SEK1 and MKK7 proteins, but the sum of the expressed proteins was almost constant in each experiment (Figs. 4–6, panels A and B).

Fig. 4 shows typical results showing the cooperative effects of SEK1 and MKK7-γ1, which appeared to be present in ES cells, on the heat shock-induced activation and phosphorylation of HA-JNK1. The expression of SEK1 or MKK7-γ1 alone clearly phosphorylated the Tyr or Thr of HA-JNK1, which was recognized with the anti-phospho-Tyr or -phospho-Thr Ab (Fig. 4, panels D and E, lanes 2 and 6). In other words, the two upstream kinases, SEK1 and MKK7-γ1, strongly favored either one amino acid or the other. The kinase activity measured by GST-c-Jun phosphorylation of HA-JNK1 was, however, rather low in either case (Fig. 4, panel G). When both Tyr and Thr residues of HA-JNK1 were phosphorylated by SEK1 and MKK7-γ1 (Fig. 4, panels D and E, lanes 3–5), there was synergistic stimulation of the kinase activity (Fig. 4, panel G). The dual phosphorylation of HA-JNK1 (Fig. 4, panel F) was also evident in the mobility shift of the Tyr-phosphorylated form on SDS-polyacrylamide gel electrophoresis (Fig. 4, panel D).

The more quantitative analysis was performed with different batches of the transfected HeLa cells, and the results are summarized in Fig. 7. As shown in Fig. 7A, SEK1 preferentially phosphorylated the Tyr residue of SAPK/JNK, whereas MKK7-γ1 mainly phosphorylated its Thr residue. These results indicate that MKK7-γ1 has a preference for the Thr phosphorylation of SAPK/JNK and that the MAPK was synergistically activated by the dual phosphorylation because of the independent actions of the two upstream MAPKKs.
obtained with LAS-1000 and are expressed as percentages of the maximum values (filled columns) including MKK7, main unknown. This gene locus also produces other isoforms.

FIG. 7. Phosphorylation of SAPK/JNK at Tyr and Thr residues in SEK1- and/or MKK7α1-, β1-, or -γ2-transfected HeLa cells. The same experiments as shown in Figs. 4–6 (corresponding to lanes 2, 4, and 6) were repeated several times. Left columns, 1 μg of SEK1, middle columns, 0.5 μg of SEK1 and 0.5 μg of MKK7α1 (panel A), -α1 (panel B), or -γ2 (panel C); right columns, 1 μg of MKK7α1 (panel A), -α1 (panel B), or -γ2 (panel C). The Tyr- (dotted columns) and Thr-phosphorylated (filled columns) levels of SAPK/JNK were measured with the Fuji LAS-1000 and are expressed as percentages of the maximum values obtained with 1 μg of SEK1 and MKK7-α2. Values shown with bars are the means ± S.E.

FIG. 8. Schematic description of MKK7 isoforms used in this study and their properties. Panel A, a schematic description of MKK7 isoforms (MKK7α1, MKK7-α1, and MKK7-α2) studied in this work. Panel B, the properties of the MKK7 isoforms. See “Results and Discussion” for explanations. TPY, Thr-Pro-Tyr motif.

tion of SAPK/JNK, appeared to be different among the isoforms (Figs. 7 and 8B). MKK7 isoforms were produced from alternative splicing of one gene locus (11). As shown in Fig. 8A, MKK7-γ1 and -γ2 but not -α1 have an NH2-terminal extension that interacts with SAPK/JNK, and MKK7-γ2 has an extra COOH-terminal region whose biochemical properties remain unknown. This gene locus also produces other isoforms including MKK7-α2, -β1, and -β2. However, apparent differences among the isoforms were not reported in the previous studies. In the present study, we found that the MKK7-γ2 isoform expressed in HeLa cells was intrinsically a “dual-specific” protein kinase for SAPK/JNK because it phosphorylated the MAPK not only at the Thr residue but also at the Tyr (Figs. 6, D and E, lane 6, and Fig. 7C). This was in sharp contrast to the action of MKK7γ1, which phosphorylated only the Thr residue of SAPK/JNK (Figs. 4, D and E, lane 6, and Fig. 7A). Moreover, MKK7α1 failed to phosphorylate SAPK/JNK at the Thr residue unless the Tyr residue of the MAPK was phosphorylated by SEK1 (Figs. 5E and 7B). These differences in MKK7 isoforms may explain the molecular mechanisms of the lost and maintained SAPK/JNK activation observed between sekl(−/−) thymocytes and peripheral T cells (see the Introduction). In other words, the SEK1-deficient phenotype would be varied in the types of cells expressing MKK7 isoforms.

Recently, Dong et al. (17) have generated mkk7(−/−) ES cells, which lack MKK7 but retain SEK1 expression. They showed that SAPK/JNK activation in response to UV irradiation, heat shock, and other stress signals was greatly reduced in the MKK7-deficient cells as had been observed in SEK1-deficient ES cells (2, 18). Thus, the two MAPKKs SEK1 and MKK7 (probably its γ1 isoform) appeared to be required for the synergistic activation of SAPK/JNK in ES cells. We have also made mkk7(−/−) bone marrow-derived mast cell lines that showed increased cell proliferation in response to the growth factors stem cell factor (c-Kit ligand) and interleukin-3. The SAPK/JNK activation by a variety of stimuli including UV irradiation and Fce receptor stimulation was lost in the mkk7(−/−) mast cells. Interestingly, the expression of SEK1 protein was strongly up-regulated in the mkk7(−/−) mast cells, and SEK1 was phosphorylated upon stimulation. These data indicate that MKK7 is essential, and SEK1 is not enough for SAPK/JNK activation in mast cells. Thus, the same synergistic SAPK/JNK activation by SEK1 and MKK7 has been observed not only in ES cells but also in differentiated mast cells.

In the present study, we mainly used heat shock as a stress signal especially in HeLa cells. The molecular mechanism by which heat shock induces SAPK/JNK activation is reported to be due to the inhibition of SAPK/JNK phosphatase(s) rather than the activation of SEK1 and MKK7 (19). This allowed us to investigate the MAPK activation without specific stimulators of the MAPKKs. Indeed, we could observe the progressive SAPK/JNK activation in a manner dependent on the expressed amounts of SEK1 and MKK7 (see Figs. 4–7). Although we have not investigated the effects of other stress signals under the same conditions, it is very likely that SAPK/JNK activation induced by those signals may be different from the present heat shock results. In relation to this, it has been reported that IL-1β and tumor necrosis factor-α mainly activate MKK7 rather than SEK1 (6, 9). In contrast, SEK1 appeared to be activated by the ββ subunits of G-proteins (20). These results indicate that SEK1- and MKK7-dependent signaling pathways exist independently in living cells. Our transfection systems presented in this study would be useful to distinguish such differences in intact cell levels.

In the stress-responsive SAPK/JNK cascade, full activation of the MAPK mostly requires both stimulations of the two separate MAPKKs, and the activated MAPK phosphorylates its downstream substrates, such as c-Jun and the members of the AP-1 transcription family, to regulate proper gene expression. Therefore, this signaling should strictly proceed without errors. It is thus tempting to speculate that the existence of the two separate pathways leading to SAPK/JNK activation may physiologically function as a fail-safe mechanism.

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