Activation Mechanism of c-Jun Amino-terminal Kinase in the Course of Neural Differentiation of P19 Embryonic Carcinoma Cells

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P19 embryonic carcinoma cells, a model system for studying early development and differentiation, can differentiate into neurons and primitive endoderm-like cells depending on the culture conditions. We have previously reported that the activation of c-Jun amino-terminal kinase (JNK) is required for the retinoic acid-induced neural differentiation of P19 cells. However, the signaling pathway(s) responsible for the activation of JNK has not been known. In this study, we demonstrated that activities of MAPK kinase 4 (MKK4) and TAK1, one of the upstream kinases of MKK4, were enhanced in the neurally differentiating cells. Inhibition of the neural differentiation by an overexpression of protein phosphatase 2Cε, an inactivator of TAK1, suggested a critical role of the TAK1 signaling pathway during the differentiation. Confocal microscopic analysis indicated that TAK1, phospho-MKK4, and phospho-JNK were colocalized with tubulin in the neurites and localized also in the nuclei of the differentiating cells. In contrast, two TAK1-binding proteins, TAB1 and TAB2, which are involved in the activation of TAK1, were localized in the neurites and the nuclei of the differentiating cells, respectively. These results suggest that two distinct TAK1-MKK4-JNK signaling pathways are independently activated at the different intracellular locations and may participate in the regulation of the neural differentiation of P19 cells.

c-Jun amino-terminal kinases (JNKs) belong to the mitogen-activated protein kinase (MAPK) superfamily and are implicated in the regulation of diverse cellular functions. JNKs are activated by a variety of stimuli, including cellular stress exerted by protein synthesis inhibitors, UV, osmotic imbalance, and proinflammatory cytokines (1, 2). Recently, the JNK signaling pathway has been implicated in a number of cellular functions unrelated to stress responses, including proliferation and differentiation (3, 4). Two MAPK kinases (MKKs), MKK4 (5, 6) and MKK7 (7, 8) have been identified as JNK activators. These MKKs, in turn, are similarly activated by the phosphorylation of conserved serine and threonine residues by MAPK kinase kinase (MKKK) family members including the MEKK family (MEKK1, MEKK2, MEKK3, and MEKK4/MTK1), MLK family (MLK1, MLK2, MLK3, and dual leucine zipper kinase (DLK)), and TAK1 and ASK1 (9–16).

P19 cells are murine embryonic carcinoma cells that have been used as a model system for studying early embryonic development and differentiation. P19 cells resemble the inner cell mass of early embryo and can differentiate to primitive endoderm-like cells, neuroectoderm-like cells, or muscle-like cells depending on the culture conditions (17–19). The treatment of aggregated P19 cells in a bacterial grade culture dish with low concentrations (10 nM) of retinoic acid (RA) followed by plating onto a tissue culture grade dish leads to differentiation to primitive endoderm-like cells, whereas the treatment of aggregated P19 cells with higher concentrations (1 μM) of RA results in their differentiation into neurons and glia (17–19).

Studies on signaling systems responsible for RA-induced primitive endodermal and neural differentiation of P19 cells have indicated that the JNK signaling pathway plays an important role (20–22). Expression of Gα12 and Gα13 subunits of the heterotrimeric G-protein is enhanced in the course of RA-induced primitive endodermal differentiation of P19 cells. Overexpression of the constitutively active form of Gα12 activated JNK and induced primitive endodermal differentiation, even in the absence of RA treatment (20). In addition, expression of a dominant negative mutant of JNK suppressed the RA-induced primitive endodermal differentiation of P19 cells, indicating that JNK plays a pivotal role in the differentiation (20). Recently, it has been reported that MEKK4 mediated JNK activation and differentiation of P19 cells to endoderm-like cells (23).

We have previously reported that the activation of JNK was also required for the RA-induced neural differentiation of P19 cells (22). However, the upstream signaling pathway responsible for the activation of JNK in the course of the neutral...
determination has not been elucidated. Here, we present evidence indicating that activity levels of MKK4 and TAK1 are enhanced in the neurally differentiating cells and that expression of protein phosphatase 2C (PP2C), an inactivator of TAK1 (24), inhibits the neural differentiation. Although TAK1, phospho-MKK4, and phospho-JNK were localized in both the nuclei and the neurites of the neurally differentiating cells, two adapter proteins involved in the activation of TAK1 were differentially localized, suggesting that two TAK1-MKK4-JNK signaling pathways are independently activated at the different intracellular locations and may participate in the regulation of the neural differentiation of P19 cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—JNK1 rabbit polyclonal antibody and MKK4 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MKK7 rabbit polyclonal antibody was a generous gift from Dr. E. Nishida (Kyoto University, Kyoto, Japan). Phospho-specific JNK rabbit antibody and phospho-specific SEK1/MKK4 rabbit antibody were purchased from New England Biolabs (Beverly, MA). Anti-TAK1, anti-TAB1, and anti-TAB2 antibodies were already described (25). Anti-α-tubulin mouse monoclonal antibody was purchased from Sigma. Cy3-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR).

**Cell Culture and Neural Differentiation**—P19 cells were cultured in α-minimum essential medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum. For neural differentiation, P19 cells were cultured in medium containing 1 μM RA in a bacterial grade culture dish for 4 days, and then the aggregates were plated onto a tissue culture grade dish as a monolayer and cultured for another 3 days in the absence of RA. For osmotic shock, undifferentiated P19 cells were incubated with medium supplemented with 0.7 mM NaCl for 15 min at 37 °C.

**Immunoprecipitation and Kinase Assay**—P19 cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 50 mM NaF, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM diithiothreitol). The cell lysates were incubated with the indicated antibodies (1 μg) at 4 °C for 1 h followed by incubation with 10 μl of protein G-Sepharose 4FF beads for 1 h. The beads were then washed three times with the lysis buffer. Activities of MKKs and JNK were determined using His-JNK5 (K55R) and GST-JNK, respectively, as the substrates (22).

**Immunoblot Analysis**—Immunoblot analysis was carried out as described previously except using the ECL system (Amersham Biosciences) to detect protein (22).

**Northern Blotting**—Total RNA (10 μg) was separated on 1.2% (w/v) formaldehyde gel and subsequently transferred to a nylon membrane. Hybridization and washing conditions were described previously (27).

**Stable Transfection**—P19 cells were co-transfected with pBABE that contains the puromycin-resistant gene and pCX-Myc empty vector or pCX-Myc-PP2Ce. Expression of Myc-PP2Ce was examined using anti-Myc antibody.

**Indirect Immunofluorescence**—For immunofluorescence, cells were cultured on a poly-l-lysine-coated cover glass (Asahi Techno Glass, Tokyo, Japan) and then fixed for 15 min with 4% (w/v) paraformaldehyde. The fixed cells were permeabilized with 0.1% (v/v) Triton X-100, blocked for 30 min in phosphate-buffered saline containing 2% (v/v) normal goat serum, and subsequently incubated with the indicated first antibodies for 1 h at room temperature or overnight at 4 °C. After extensive washing with phosphate-buffered saline, the cells were incubated with the secondary antibody for 1 h at room temperature. After repeated washing with phosphate-buffered saline, the cover glass was prepared for immunofluorescence by mounting in VECTASHIELD (Vector Laboratories, Burlingame, CA). The cells were examined by confocal microscopy with a Zeiss LSM410 microscope (Carl Zeiss, Jena, Germany).

**RESULTS**

**MKK4 Is Selectively Activated in the Course of RA-induced Neural Differentiation of P19 Cells**—We have previously shown that the activation of JNK was required for the RA-induced neural differentiation of P19 cells using a dominant negative mutant of JNK (22). However, the mechanism of the JNK activation was not clear. Therefore, we investigated the upstream signaling pathways of JNK in the P19 cells before and after the induction of neural differentiation. We compared the activities of MKK4 and MKK7, which are known to be the physiological activators of JNK, of the neurally differentiating cells with those of the undifferentiated or stress-treated cells. To determine the MKK4 and MKK7 activities of cell extracts, an immune complex kinase assay was performed using a kinase-negative mutant of JNK (His-JNK (K55R)) as the substrate. Interestingly, only MKK4 was substantially activated in the neurally differentiating cells, whereas both MKK4 and MKK7 were activated in response to stress (Fig. 1, a and b). MKK4 itself is known to be activated through phosphorylation by upstream kinases. Concomitant with the activation of MKK4, an increase in phosphorylation of MKK4 in the neurally differentiating cells was observed (Fig. 1d). In parallel with the activation of MKK4, JNK was also activated during the neural differentiation, confirming our previous observation (Fig. 1a) (22). The degree of the activation of JNK in the neurally differentiating cells was similar to that induced by the addition of NaCl to the medium. These results indicated that MKK4, but not MKK7, was the major activator of JNK during the neural differentiation.

**TAK1 Is Activated in the Neurally Differentiating Cells**—We were interested in identifying the MKKK that is responsible for the phosphorylation and activation of MKK4 in the neurally differentiating cells. Recently, a number of MKKKs that phosphorylate and activate MKK4 have been reported (9–16). Using Northern blot analysis, we first examined whether the mRNA levels of MEKK1, MEKK3, MEKK4/MTK1, ASK1, and TAK1 increased in the course of the differentiation. The results
Fig. 2. TAK1 is activated in the neurally differentiating P19 cells. a, total RNA was prepared from the duplicated dishes of the undifferentiated P19 cells (indicated as Undif.) and the neurally differentiating cells (indicated as Neural). RNA was separated on an agarose gel containing formaldehyde, transferred to nylon membrane, and probed with \(^{32}\)P-labeled cDNAs as indicated (upper panels). The membranes were deprobed and reprobed with \(^{32}\)P-labeled cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to indicate equal loading of RNA samples (lower panels). b, aliquots of the cell extracts (0.5 mg of protein) from the undifferentiated (lanes 1 and 3) and the neurally differentiating cells (lanes 2 and 4) were immunoprecipitated (IP) with normal rabbit IgG (indicated by IgG) or anti-TAK1 antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-TAK1 (upper panel) and anti-TAB1 (lower panel) antibodies. WB, Western blot; c, the lysates (10 µg) from the undifferentiated and neurally differentiating cells were analyzed by immunoblotting with anti-TAB2 antibody. d, the immunoprecipitates obtained from the neurally differentiating cells (panel b, lane 4) were incubated without (+) or with (+) λ-phosphatase (λ-phos) for 1 h and then immunoblotted with anti-TAB1 antibody (left panel). The lysates (10 µg) of the neurally differentiating cells were subjected to SDS-PAGE and analyzed by immunoblotting with anti-TAB2 antibody before and after λ-phosphatase treatment (right panel).

demonstrated that there was little difference in the expression levels of MEKK1, MEKK3, MEKK4/MTK1, and ASK1 between the undifferentiated cells and the neurally differentiating cells (Fig. 2a). In contrast, the expression level of TAK1 was substantially increased in the neurally differentiating cells (Fig. 2a). Therefore, we determined whether the protein level of TAK1 was also increased in accordance with the neural differentiation. TAK1 was immunoprecipitated with anti-TAK1 antibody from the cell lysates of undifferentiated and neurally differentiating cells. Immunoblot analysis using the anti-TAK1 antibody demonstrated that the TAK1 protein level was indeed enhanced in the neurally differentiating cells (Fig. 2b, upper panel). It has been established that TAB1, a TAK1-binding protein, associates with TAK1 and induces the activation of TAK1 by autophosphorylation in the cells (25). Upon the activation of TAK1, TAB1 is phosphorylated. It is also known that TAB2, another TAK1-binding protein that mediates interleukin-1-induced signaling to TAK1, is also phosphorylated concomitant with the activation of TAK1 in vivo (28). The phosphorylation of the endogenous TAB1 by TAK1 can be monitored by its mobility shifts on Western blot analysis. Using this method, we determined whether phosphorylation of TAB1 by TAK1 is enhanced in the neurally differentiating cells. TAK1/TAB1 complex was first immunoprecipitated from the cell extracts with anti-TAK1 antibody and probed with anti-TAB1 antibody on immunoblot analysis (Fig. 2b, lower panel). An enhanced mobility shift of TAB1 was observed in the neurally differentiating cells, and this mobility shift was cancelled by the incubation of the immunoprecipitates with bacterial λ-phosphatase (Fig. 2d, left panel), indicating that the mobility shift was indeed caused by the enhanced phosphorylation of TAB1. Similar to TAB1, an increased mobility shift of TAB2 of the neurally differentiating cells was observed, which was also cancelled by the phosphatase treatment of the cell extracts (Fig. 2, c and d). Collectively, these observations indicate that TAK1 was activated in the neurally differentiating cells.

**PP2Ce Suppresses the Neural Differentiation of P19 Cells**—We have recently reported that PP2Ce, a member of protein phosphatase 2C family, inactivated the SAPK signaling pathway by selectively associating with and dephosphorylating TAK1 (24). PP2Ce gave no influence on the phosphorylation levels of the downstream components of TAK1 such as M KK4 and JNK. To test whether the activation of the TAK1 signaling pathway is required for the neural differentiation of P19 cells, we expressed PP2Ce stably in P19 cells and determined the effect of PP2Ce expression on the differentiation. The results demonstrated that the neural differentiation was markedly suppressed by the expression of PP2Ce (Fig. 3, a and b). Interestingly, expression level of endogenous PP2Ce mRNA was substantially enhanced at day 4 (aggregation culture) of the RA-induced neural differentiation process of P19 cells, and the expression was further enhanced at day 7 (Fig. 3c).

**TAB1 and TAB2 Are Differentially Localized in the Neurally Differentiating Cells**—We performed confocal microscopic analysis to determine the subcellular localization of phospho-JNK and phospho-MKK4 in the cells before and during neural differentiation. Both phospho-JNK and phospho-MKK4 were localized exclusively in the nucleus in the undifferentiated P19 cells (data not shown). In contrast, in the neurally differentiating cells, both the phospho-JNK and the phospho-MKK4
were colocalized with tubulin in the neurites (Fig. 4, a and b). Both of them were also detected in the nuclei, although the signals of phospho-JNK in the nuclei were much weaker than those in the neurites.

Studies of subcellular localization of TAK1, TAB1, and TAB2 in the neurally differentiating cells revealed that, whereas TAK1 was colocalized with tubulin in the neurites and also localized in the cell nuclei (Fig. 4c), TAB1 was predominantly colocalized with tubulin in the neurites (Fig. 4d), and TAB2 was mainly localized in the nuclei of the neurally differentiating cells (Fig. 4e).

**DISCUSSION**

In this study, we investigated the signaling pathway responsible for the sustained activation of JNK, which is required for the RA-induced neural differentiation of P19 cells. We observed the enhanced activities of JNK, MKK4, and TAK1 in the neurally differentiating cells (Figs. 1 and 2). All of these proteins were colocalized with tubulin in the neurites and also localized in the cell nuclei (Fig. 4). These observations suggest that the TAK1-MKK4 signaling pathway participates in the activation of JNK in the course of neural differentiation of P19 cells. Recently, we have shown that PP2Ce interacted selectively with TAK1 and inactivated it by dephosphorylation (24). PP2Ce did not dephosphorylate downstream components of TAK1 such as MKK4/7 and JNK/p38. These results suggested that PP2Ce acts as a specific inactivator of TAK1 in the SAPK signaling pathway. Inhibition of the neural differentiation of P19 cells by overexpression of PP2Ce has been reported to follow incubation with Cy3-conjugated goat anti-rabbit IgG antibody. The cells were double-stained with anti-tubulin antibody followed by incubation with Alexa 488-conjugated goat anti-mouse IgG antibody. Hoechst 33258 dye was used to show the nuclei. The scale bar indicates 25 μm.

Interestingly, two endogenous TAK1-binding proteins were differently localized in the neurally differentiating cells. Thus, TAB1 was colocized with tubulin in the neurites, and TAB2 was mainly localized in the cell nuclei (Fig. 4, d and e). TAB1 and TAB2 have been reported to participate in the tumor growth factor-β family and interleukin-1-induced activations of TAK1, respectively (15, 29). Both the membranous and the nuclear localizations of TAB2 have been reported previously (30, 31). Therefore, the different subcellular localization of TAB1 and TAB2 in the neurally differentiating cells may indicate that JNKs in the neurites and the cell nuclei are independently activated by two different TAK1 signaling pathways, each activated by different extracellular stimulus.

The sustained activation of JNK has also been reported in the differentiating cerebellar granule neurons of rats, although both MKK4 and MKK7 were activated in this case (32). Similar to the case of the neurally differentiating P19 cells, JNK was localized both in neurites and in nuclei of the differentiating cells.

**FIG. 3.** PP2Ce suppresses the neural differentiation of P19 cells. a, empty vector (lane 1) or expression plasmid of Myc-tagged PP2Ce (lane 2) was stably transfected to P19 cells, and the expressed proteins were detected with anti-Myc antibody. b, P19 stable transfectants (left panel, empty vector; right panel, PP2Ce expression vector) were treated with 1 μM RA and immunostained with anti-tubulin antibody. c, P19 cells (day 0) were cultured in the absence or presence of 1 μM RA on a bacterial grade culture dish for 4 days and then grown on a tissue culture grade dish containing medium without RA for another 3 days. Total mRNAs were prepared at days 0, 4, and 7, and Northern blot analysis was carried out using PP2Ce cDNA (upper panel) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lower panel) as the probe.

**FIG. 4.** Subcellular localization of phospho-JNK, phospho-MKK4, TAK1, TAB1, and TAB2 in the neurally differentiating P19 cells. a–e, P19 cells during neural differentiation were fixed with paraformaldehyde, and the proteins were detected using specific antibodies as indicated followed by incubation with Cy3-conjugated goat anti-rabbit IgG antibody. The cells were double-stained with anti-tubulin antibody followed by incubation with Alexa 488-conjugated goat anti-mouse IgG antibody. Hoechst 33258 dye was used to show the nuclei. The scale bar indicates 25 μm.
cerebellar granule neurons. These observations raise the possibility that the cytosolic JNK plays a unique role in the neural differentiation. In this context, it was reported that JNK phosphorylated cytoskeletal proteins, such as neurofilament heavy chain and tau protein in the neural cells (33). The activated JNK in the neurites may participate in the regulation of neurite formation through regulation of the cytoskeletal proteins by phosphorylation.

P19 cells differentiate to primitive endodermal-like cells on treatment with lower concentrations of RA and, similar to the neural differentiation, the activation of JNK is required for the primitive endodermal differentiation. Overexpression of constitutively active mutants of α12/α13 subunits of the heterotrimeric G-protein (Gα12(Q229L) and Gα13(Q226L)) in P19 cells also induced the primitive endodermal differentiation (20). Wang et al. (36) showed that MKK4 was constitutively activated in the P19 cells stably expressing Gα12(Q229L) protein. We have also shown that MKK4 but not MKK7 was activated in the course of the RA-induced primitive endodermal differentiation of P19 cells (26). These observations suggest that the activation of MKK4 but not MKK7 serves as the common mechanism of JNK activation in both the neural and the primitive endodermal differentiation of P19 cells. However, studies of the signaling pathway upstream of MKK4 in the primitive endodermally differentiating P19 cells have indicated that MEKK4 was responsible for the activation of MKK4 (23). Therefore, the mechanism of the activation of MKK4 may differ depending on the direction of differentiation. Further studies of the signaling pathways upstream of MKK4s in neurally and primitive endodermally differentiating cells will contribute to the understanding of the molecular mechanisms underlying the multipotency of P19 cells.

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