MEKK4 Mediates Differentiation in Response to Retinoic Acid via Activation of c-Jun N-terminal Kinase in Rat Embryonal Carcinoma P19 Cells*

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Differentiation of P19 embryonal carcinoma cells in response to the morphogen retinoic acid is regulated by Gαi2/13 and is associated with activation of c-Jun N-terminal kinase. The role of MEKK1 and MEKK4 upstream of the c-Jun N-terminal kinase was investigated in P19 cells. P19 clones stably expressing constitutively active and dominant negative mutants of MEKK1 and MEKK4 were created and characterized. Expression of the constitutively active form of either MEKK1 or MEKK4 mimicked the action of retinoic acid, inducing these embryonal carcinoma cells to primitive endoderm. Expression of the dominant negative form of MEKK1 had no influence on the ability of retinoic acid to induce either JNK activation or primitive endoderm formation in P19 stem cells. Expression of the dominant negative form of MEKK4, in contrast, effectively blocks both morphogen-induced activation of JNK and cellular differentiation. These data identify MEKK4 as upstream of c-Jun N-terminal kinase in the pathway mediating differentiation of P19 stem cells to primitive endoderm.

A central role for heterotrimeric G-protein-mediated signaling in cell proliferation, differentiation, and apoptosis has been established (1). Various G-proteins have been shown to regulate features of cellular differentiation. Goα suppresses adipogenesis of the NIH 3T3-L1 embryonal fibroblasts, which can be relieved by loss of Goα in response to inducers of differentiation and mimicked with oligodeoxynucleotides antisense to Goα (2). Activation of Goα via cholaer intoxication, in contrast, blocks induction of adipogenesis (2). Overexpression of wild-type Goα or its Q205L constitutively activated mutant can induce differentiation in these 3T3-L1 cells (3). Expression of the highly abundant G-protein subunit Goβ has been shown to be oblige for induction of growth cones in developing neurites of nerve growth factor-treated PC12 cells (4, 5). For Goα13, which suppresses phospholipase C activity in the F9 teratocarcinoma cells (6), morphogens stimulate a decline in Goα13, which de-represses phospholipase C (7) and stimulates the generation of inositol phosphates and diacylglycerol, which in turn activates protein kinase C (8). The activation of protein kinase C and the ERK1/2 members of the mitogen-activated protein kinase family is the basis for morphogen-induced differentiation of these totipotent F9 cells (9). Expression of wild-type or a lesser amount of the constitutively activated Q205L mutant form of Goα13 blocks induction of F9 cell differentiation (10). Retroviral infection with a vector expressing RNA antisense to Goα2, but not one expressing RNA antisense to either Goα or Goα, provokes a decline in Goα2 and differentiation to primitive endoderm in the absence of morphogen (7). Heterotrimeric G-proteins have been implicated as mediators of early stages of vertebrate development in frogs and zebrafish (11–13). Finally, several inherited diseases that alter the expression or activity of heterotrimeric G-protein α subunits display profound effects on human development (14, 15).

The P19 embryonal carcinoma cells provide a useful model for murine pre-implantation development (16, 17). Three germ layers, endoderm, mesoderm, and ectoderm, can be derived from P19 cells through the use of appropriate culture conditions and an inducer(s) (18). Retinoic acid stimulates P19 cells to differentiate into primitive endoderm, as defined by the loss of the embryonic antigen SSEA-1 and positive staining with the TRMIA antibody, specific for the cytokeratin endo A protein that is the hallmark of the endodermal phenotype (19). RA-induced differentiation is accompanied by the expression of Goα13 and activation of JNK, but neither ERK1/2 or p38 members of the mitogen-activated protein kinase family (20, 21). Expression of the constitutively active mutant form of Goα13 activates JNK and induces P19 clones to differentiate into primitive endoderm in the absence of RA induction. In addition, expression of the dominant negative form of JNK1 blocks the ability of the morphogen to induce the cells to primitive endoderm (20). In an effort to elucidate the linkage from Goα13 to JNK activation in P19 cell differentiation, we explored the upstream control of JNK activation, focusing on the potential roles of two mitogen and extracellularly activated protein kinase kinases, MEKK1 and MEKK4 (22, 23). The results provide compelling evidence that MEKK4 mediates the signaling from retinoic acid to JNK and differentiation in these embryonic carcinoma cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—The P19 embryonal carcinoma cells were purchased from the American Type Culture Collection (Ma-
Expression of wild-type and constitutively active MEKK1 induces differentiation in P19 embryonal carcinoma cells. Cells were transfected with WT, CA, and DN forms of MEKK1 that were N-terminal-tagged with the HA peptide. Stably transfected clones were analyzed for the level of expression of the proteins, JNK1 activity, and phenotypic changes. A, whole-cell lysates were resolved on a 10% acrylamide gel by SDS-PAGE, and proteins were transferred to nitrocellulose membrane. The immunoblot was probed with a polyclonal antibody raised against the HA peptide. Solid-phase JNK1 assay was performed on immunoprecipitates obtained by immunoprecipitating the cell lysate on protein A/G-agarose beads using a polyclonal antibody to JNK1. Bacterially expressed recombinant protein, GST-c-Jun, was employed as a substrate. The phosphorylation product was analyzed on a 10% acrylamide gel by SDS-PAGE and processed for autoradiography. A replicate of the immunoprecipitates was used to detect the level of JNK1 protein by immunoblotting followed by probing with a polyclonal antibody to JNK1. The data shown are representative of at least three independent experiments for each. B, cells overexpressing different forms of MEKK1 were cultured in chamber slides and processed for immunofluorescence using an antibody to cytokeratin endo-A (TROMA-1), the marker protein for primitive endoderm. Phase-contrast (PC) and indirect immunofluorescence (IIF) images are shown. EV, empty vector.
MEKK4 Dominant Negative Blocks Differentiation

**RESULTS**

Rat embryonic carcinoma P19 cells were stably transfected with the expression vectors harboring WT, CA, and DN mutant forms of either MEKK1 or MEKK4 (23). Endogenous MEKK1 and MEKK4 were identified in immunobLOTS of extracts of P19, mouse embryonic fibroblast 3T3-L1, and human epidermoid carcinoma cells subjected first to SDS-PAGE (Fig. 1A). MEKK4 is subject to ready proteolysis with a terminal, long lived fragments displaying molecular masses of ~100 kDa and ~115 kDa, as observed in P19 and 3T3-L1 cells. The full-length form is observed in the blot of A431 cells shown here. MEKK4, likewise, is cleaved to a terminal, long lived fragment displaying a molecular mass of ~100 kDa in blots prepared from P19, 3T3-L1, and A431 cells. Thus, both MEKK1 and MEKK4 are present in P19 cells, and both kinases are sensitive to proteolytic cleavage. These cleavages of MEKK family members are believed to be a normal and necessary processing step in their biological activation (26).

HA-tagged forms of MEKK1 WT and CA and DN mutant forms were stably expressed, and extracts of these clones were subjected to immunoblotting with antibodies specific for the immunoprecipitates using the recombinant GST-c-Jun as a substrate. The kinase assay products were analyzed on a 10% acrylamide gel by SDS-PAGE. A replicate of the immunoprecipitates was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Immunodetection of the JNK1 was performed using an antibody to JNK1. The gels were stained, dried, and subjected to autoradiography. B. cells cultured simultaneously in chamber slides were fixed and processed for indirect immunofluorescence detection of phenotype-specific marker proteins. Endodermal marker, cytokeratin endo A (TROMA-1), and embryonal marker SSEA-1 were detected by indirect immunofluorescence. Both phase contrast (PC) and epifluorescence (IIF) images are shown.

**FIG. 3.** Differentiation of P19 embryonal carcinoma cells in response to expression of constitutively active MEKK1 is not enhanced further by retinoic acid. P19 clones overexpressing constitutively active forms of MEKK1 and clones harboring the "empty" expression vector were treated with retinoic acid (10 nM) for 2 days while cultured in aggregates in bacterial grade Petri dishes. Treatment of retinoic acid was conducted for 2 additional days after seeding the cells in tissue culture grade Petri dishes. A aliquots of the cell suspensions were seeded also in chamber slides. A, cells treated with retinoic acid for 4 days were harvested, and crude cell lysate was prepared. JNK1 was immunoprecipitated from the lysates using antibody to JNK1. Solid-phase kinase assay was performed on the immunoprecipitates using the recombinant GST-c-Jun as a substrate. The kinase assay products were analyzed on a 10% acrylamide gel by SDS-PAGE. A replicate of the immunoprecipitates was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Immunodetection of the JNK1 was performed using an antibody to JNK1. The gels were stained, dried, and subjected to autoradiography. B, cells cultured simultaneously in chamber slides were fixed and processed for indirect immunofluorescence detection of phenotype-specific marker proteins. Endodermal marker, cytokeratin endo A (TROMA-1), and embryonal marker SSEA-1 were detected by indirect immunofluorescence. Both phase contrast (PC) and epifluorescence (IIF) images are shown.
FIG. 4. Retinoic acid induces differentiation of P19 cells overexpressing a dominant negative form of MEKK1. P19 clones harboring empty expression vector and clones overexpressing the DN form of MEKK1 were analyzed for JNK1 activity of whole-cell lysates and for phenotype. Cells were treated with 10 nM RA, as aggregates seeded on bacterial-grade Petri dishes. After 2 days of treatment, the aggregates were dissociated and cultured on tissue culture dishes for an additional period of 2 days in the presence of RA (+RA). A, whole-cell lysates were prepared from each set of conditions. JNK1 was immunoprecipitated from the lysates using antibody to JNK1. Solid-phase kinase assay was performed on the immunoprecipitates using the recombinant GST-c-Jun as a substrate. The kinase assay products were analyzed on a 10% acrylamide gel by SDS-PAGE. A replicate of the immunoprecipitates was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Immunodetection of the JNK1 was performed using an antibody to JNK1. The gels were stained, dried, and subjected to autoradiography. B, aliquots of the cell suspensions were cultured simultaneously in chamber slides. At the end of the retinoic acid treatment, cells were harvested for further analysis. Cells grown in chamber slides were fixed and processed for indirect immunofluorescence using an antibody (TROMA) to the endodermal marker antigen, cytokeratin endo A. A duplicate set of cells was immunostained with an antibody to an embryonic marker-specific antigen, SSEA-1. Both phase contrast and epifluorescence images are displayed. The results are representative of at least three separate experiments. PC, contrast images; IIF, epifluorescence images; EV, empty vector.
HA epitope (Fig. 1B). Much like that observed for the endogenous MEKKs, MEKK1 and MEKK4 wild-type and mutant forms were subject to proteolytic cleavage to a limit digest of ~100 kDa. Multiple clones were prepared and characterized for stable transfection of each construct. The immunoblotting reveals similar levels of expression for wild-type and mutant forms of MEKK1 and for those of MEKK4, although the level of expression for MEKK1 was consistently greater than that observed for MEKK4. The identity of the stained species of wild-types MEKK1 and MEKK4 as HA-tagged was confirmed by use of the HA-blocking peptide (Fig. 1C). In the presence of the HA antigen, the staining of the HA-tagged MEKK1 and MEKK4 forms was virtually abolished. Similar experiments performed with the HA-tagged mutant versions of either MEKK displayed the same sensitivity to blocking by the HA antigen peptide (data not shown).

The various forms of MEKK1 and MEKK4 expressed in P19 cells were examined for their ability to phosphorylate a common substrate. The HA-tagged mutant MEKKs were immunoprecipitated from whole-cell lysates from P19 clones, and their ability to catalyze the phosphorylation of the substrate, rGST-JNKK1 protein, was evaluated for multiple clones from each of the stable transfections (Fig. 1D). The results of representative assays reveal that the expression of the DN forms of either MEKK resulted in a blunted phosphorylation of rGST-JNKK1 in comparison with that of extracts from cells expressing the WT forms of each MEKK. Expression of the constitutively activated forms of MEKK1 and MEKK4 provoked a sharp increase in the phosphorylation of their downstream substrate, JNK1.

Assay of JNK activity in the whole-cell extracts of the clones expressing WT, CA, or DN forms of MEKK1 using a GST-c-Jun fusion protein as a read-out confirms the increased activation of JNK1 in the clones overexpressing WT-MEKK1 or expressing the CA-MEKK1 (Fig. 2A). JNK1 activity increased ~3-fold in the clones overexpressing CA-MEKK1 as well as in the clones expressing WT-MEKK1. The P19 clones expressing the DN form of MEKK1 displayed a low level of JNK1 activity, similar to that of the clones harboring the empty expression vector. Most interestingly, expression of CA-MEKK1 was sufficient to induce differentiation of P19 cells to an endodermal phenotype in the absence of the morphogen RA, as evidenced by positive staining with the TROMA antibody (Fig. 2B). The positive staining of P19 clones expressing CA-MEKK1 was similar to that of the P19 clones transfected with empty vector alone that had been induced to differentiate to primitive endoderm with RA. The expression of the WT-MEKK1 also induced the P19 clones to differentiate and stain positive for TROMA. In sharp contrast to the clones expressing either WT-MEKK1 or CA-MEKK1, those expressing DN-MEKK1 display no positive staining for TROMA. Staining of the SSEA-1 embryonic antigen was negative for clones expressing WT-MEKK1 or CA-MEKK1 and positive for the clones expressing the DN-MEKK1 (not shown).

The effects of addition of RA to the clones expressing the CA form of MEKK1 on JNK activation and on the differentiation process was examined (Fig. 3). RA alone stimulates a 2–3-fold increase in the activity of JNK1, as measured using the GST-c-Jun fusion protein as a substrate. The expression of the CA form of MEKK1 yields about the same level of activation of JNK1. Challenging the clones that express the CA-MEKK1 with the morphogen RA failed to increase the level of JNK1 activity beyond that observed with either CA-MEKK1 expression or RA treatment alone, suggesting that the effects were not additive. In each of the trials, RA treatment of the clones expressing CA-MEKK1 resulted in a modest (<20%) decline in JNK1 activity. Companion studies of indirect immunofluorescence microscopy of the clones expressing the CA form of MEKK1 reveal the absence of the SSEA-1 antigen and the strong, positive staining of the endodermal marker antigen with TROMA for both the clones expressing CA-MEKK1 in the absence or presence of RA (Fig. 3B). These data agree well with the measurements of JNK1 activity and suggest that the effects for RA and expression of CA-MEKK1 in the differentiation of the P19 cells are non-additive in nature.

In view of the ability of overexpression of MEKK1 or expression of the CA-MEKK1 to induce differentiation much like RA does, the effects of expression of the DN form of MEKK1 on the ability of RA to induce differentiation was investigated (Fig. 4). Measurements of JNK1 activity using the GST-c-Jun fusion protein as a substrate show the ability of the DN-MEKK1 to suppress JNK activity in the cells unchallenged with morphogen while not preventing the ability of RA to induce a 4-fold activation of JNK1 (Fig. 4A). RA induces a loss in staining of SSEA-1 and an increase in TROMA staining in the clones harboring the expression vector, which is “empty” (EV), in good
agreement with the results obtained with wild-type P19 cells. Interestingly, the expression of the dominant negative form of MEKK1 failed to influence the ability of RA to differentiate the P19 clones to primitive endoderm. The clones expressing DN-MEKK1 lost SSEA-1 staining and displayed positive staining by TROMA in response to RA. Although overexpression of WT-MEKK1 and expression of CA-MEKK1 can cause the P19 stem cells to differentiate, mimicking the effects of RA itself, MEKK1 does not appear to play an obligate role in the signaling of this process.

Of the other major upstream regulator of JNKs (23), MEKK4 was explored for its possible role in RA-induced differentiation of P19 cells. Analysis of the JNK1 activity of the whole-cell extracts from clones expressing WT and CA forms of MEKK4 reveals a 2.5-fold and a 4.0-fold increase in JNK1 activity, respectively, using the GST-c-Jun fusion protein as a substrate for phosphorylation. The measurements of JNK1 activity agree well with the phenotypic changes in the clones overexpressing WT-MEKK4 or expressing CA-MEKK4 (Fig. 5A). The clones expressing the DN mutant of MEKK4 displayed low JNK1 activity, as predicted from the dominant negative nature of the MEKK4 construct. Much like the effects of overexpression of the wild-type form and expression of the constitutively active form of MEKK1, the analogous forms of MEKK4 were found to provoke differentiation of P19 clones (Fig. 5B). Based upon positive staining of the endodermal marker TROMA, clones overexpressing WT-MEKK4 and CA-MEKK4 differentiate in the absence of the morphogen RA, displaying cell morphologies and staining patterns not unlike the P19 clones harboring the empty vector upon treatment with RA. The clones expressing the DN form of MEKK4, in sharp contrast to those expressing either WT- or CA-MEKK4, displayed no TROMA staining.

The clones expressing the CA-MEKK4 were challenged with RA in an effort to ascertain if the differentiation process was sensitive to the morphogen (Fig. 6). The measurements of JNK1 activity reveal a 2.0-fold increase in phosphorylation of the GST-c-Jun fusion protein with samples from the P19 clones harboring the empty vector following challenge with RA (Fig. 6A). The expression of CA-MEKK4 was accompanied by a 3.5-fold increase in JNK1 activity, correlating well with the differentiation of these cells to primitive endoderm. When the clones expressing the CA-MEKK4 were challenged with RA, the amount of JNK1 activity displayed a reproducible, modest decline. This modest decline in JNK1 activity in response to RA for the clones expressing CA-MEKK4 was similar to the decline also noted in the clones expressing CA-MEKK1 following challenge with RA. Constitutively active MEKK4 provokes differentiation to primitive endoderm and prominent staining by the TROMA antibody. SSEA-1 staining is absent in the clones expressing CA-MEKK4, confirming the loss of the embryonic state and differentiation to endoderm (Fig. 6B). When treated with RA, the clones expressing CA-MEKK4 appeared much like the clones not exposed to RA, with the phenotypic mor-

FIG. 6. Differentiation of P19 embryonal carcinoma cells in response to expression of constitutively active MEKK4 is not enhanced further by retinoic acid. P19 clones overexpressing constitutively active forms of MEKK4 and clones harboring the “empty” expression vector were treated with retinoic acid (10 nM) for 2 days while cultured in aggregates in bacterial grade Petri dishes. Treatment of retinoic acid was conducted for 2 additional days after seeding the cells in tissue-culture grade Petri dishes. Aliquots of the cell suspensions were seeded also in chamber slides. A, cells treated with retinoic acid for 4 days were harvested, and crude cell lysate was prepared. JNK1 was immunoprecipitated from the lysates using an antibody to JNK1. Solid-phase kinase assay was performed on the immunoprecipitates using the recombinant GST-c-Jun. The kinase assay products were analyzed on a 10% acrylamide gel by SDS-PAGE. The gels were stained, dried, and subjected to autoradiography. A replicate of the immunoprecipitates was resolved on a 10% acrylamide gel (SDS-PAGE), then transferred to nitrocellulose membrane. Immunodetection of the JNK1 was performed using an antibody to JNK1. B, cells cultured simultaneously in chamber slides were fixed and processed for indirect immunofluorescence detection of phenotype-specific marker proteins. Endodermal marker, cytokeratin endo A (TROMA-1) and embryonal marker SSEA-1 were detected by indirect immunofluorescence. Both phase contrast (PC) and epifluorescence (IIF) images are shown.
MEKK4 Dominant Negative Blocks Differentiation

Fig. 7. P19 cells overexpressing dominant negative MEKK4 fail to differentiate in response to retinoic acid. P19 clones harboring empty expression vector and clones overexpressing the DN form of MEKK4 were analyzed for JNK1 activity of whole-cell lysates and phenotype. Cells were treated with 10 nM RA as aggregates seeded on bacterial-grade Petri dishes. After 2 days of treatment, the aggregates were dissociated and cultured on tissue culture dishes for an additional period of 2 days in the presence of RA (+ RA). A, whole-cell lysates were prepared from each set of conditions. JNK1 was immunoprecipitated from the lysates using antibody to JNK1. Solid-phase kinase assay was performed on the immunoprecipitates using the recombinant GST-c-Jun as a substrate. The kinase assay products were analyzed on a 10% SDS-PAGE, then transferred to nitrocellulose membrane. Immunodetection of the JNK1 was performed using an antibody to JNK1. 

**B**

MEKK4(DN) + RA

PC

Sea

TROMA

IIF

PC

IIF

DISCUSSION

Heterotrimeric G-proteins play a pivotal role in control of differentiation and development (1). Ample examples have accumulated providing a compelling linkage between specific G-protein subunits and the ability of cells to proliferate, differentiate, and apoptose. Goi acts as a repressor to adipogenesis in a manner that does not involve the activation of adenylylcyclase (2, 27). Suppression of Goi by classical inducers or by treatment with antisense oligodeoxynucleotides leads to adipogenesis (2). Goi is expressed at high levels in the leading edge of neurite growth cones (4, 5), which collapse when Goi levels are suppressed by antisense oligodeoxynucleotides (28). Expression of the constitutively activated form of Goi promotes a doubling in neurite outgrowth in PC12 cells (29). Goi2 is critical in the differentiation of F9 teratocarcinoma stem cells to primitive endoderm (7). Overexpression of Goi2 blocks the ability of RA to induce differentiation (9, 10). RA provokes a rapid transient decline in Goi2, which when mimicked by antisense RNA also leads to differentiation in the absence of the morphogen (8).

In the current work we extended earlier studies that demonstrated a role for Go12/13 in the differentiation of P19 embryonal carcinoma cells (21). The expression of Go12/13 increased dramatically in response to RA in these cells as well as in early mouse development (21). Expression of constitutively activated Go12/13 provokes differentiation of P19 cells and downstream activation to the level of mitogen-activated protein kinases (20). JNK, but neither ERK1/2 nor p38 kinases, are activated in response either to RA or to expression of the constitutively active Go13. In addition, expression of DN-JNK1 was able to block differentiation of these cells to RA (20). Prominent upstream regulators of JNK include MEKK1 and MEKK4 (23). We demonstrate the ability of overexpression of the wild-type MEKK1 and MEKK4 or their constitutively active mutant forms to provoke a robust differentiation of the P19 cells in the absence of RA. The effects of the CA forms of either MEKK1 or MEKK4 on the ability of the P19 cells to respond to RA. Unlike the effects observed in the clones expressing the DN form of MEKK1, the expression of the DN-MEKK4 was associated with a loss in the ability of the morphogen both to activate JNK and to induce differentiation of the P19 clones (Fig. 7). Expression of the DN form of MEKK4 potently suppressed JNK activity, well below the ambient levels of JNK in the clones harboring the empty vector and in the absence of morphogen (Fig. 7A). Clones expressing the dominant negative form of MEKK4 display positive staining with antibodies for SSEA-1 embryonic antigen (Fig. 7B), indicating that the clones maintained an embryonic phenotype, even in the face of a challenge with the morphogen RA. TROMA staining of the clones expressing DN-MEKK4 was negative, also reflecting the embryonic phenotype of these clones made resistant to the actions of RA through loss of MEKK4-mediated signaling.

More revealing were the investigation of the ability of DN forms of MEKK1 and MEKK4 to influence the action of RA in these stem cells. Expression of DN-MEKK1 was without effect. Although the DN-MEKK1 displayed the ability to reduce the basal level of JNK activity in the P19 cells, it failed to block the and epifluorescence images are displayed. The results are representative of at least three separate experiments. Both phase contrast (PC) and epifluorescence (IIF) images are shown.
ability of RA to stimulate activation of JNK as well as the ability of RA to block primitive endoderm formation. Despite the fact that the CA mutant form of MEKK1 can both activate JNK and differentiation, these data provide compelling evidence that MEKK1 is not a dominant mediator of RA-induced JNK activation in the pathway controlling cellular differentiation. Expression of DN-MEKK4, in sharp contrast, blocks the ability of RA to induce activation of JNK as well as to induce differentiation of P19 cells to primitive endoderm. Interestingly, the level of expression of the MEKK1 was uniformly greater than MEKK4, as measured by staining for the HA antigen common to both. Thus, the inability of DN-MEKK1 to block RA-induced activation of JNK and differentiation cannot be ascribed to insufficient levels of expression. These studies suggest a central role for MEKK4 in the ability of RA to induce cellular differentiation via JNK. Despite the ability of both CA-MEKK1 and CA-MEKK4 to promote differentiation, only the DN form of MEKK4 specifically blocks the induction of differentiation in response to RA. The ability of the DN-MEKK4 to block RA-induced JNK activation and formation of primitive endoderm might suggest that other upstream regulators of JNK, such as MEKK2, MEKK3, and the mixed-lineage group of MEKKs (MLK) (30), do not play a dominant role in RA action to the level of JNK activation or cellular differentiation in P19 cells. The molecular basis by which the DN-MEKK4 blocks the action of retinoic acid on cellular differentiation, however, may include a variety of mechanisms, including competition for scaffold proteins as well as for molecules downstream and/or upstream of MEKKs. Further studies will be required to delineate more precisely the role of MEKK4 and the linkage between G0,13 and MEKK4 in this model of early mouse development.

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