Expression of Ga13 (Q226L) Induces P19 Stem Cells to Primitive Endoderm via MEKK1, 2, or 4*

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Ga13 mediates the ability of the morphogen retinoic acid to promote primitive endoderm formation from mouse P19 embryonal carcinoma stem cells, a process that includes the obligate activation of Jun N-terminal kinase. Expression of the constitutively activated (Q226L) GTPase-deficient form of Ga13 mimics retinoic acid and was used to investigate the signaling upstream of primitive endoderm formation. Jun N-terminal kinase 1 activity, MEK1,2, MKK4, and MEKK1 were constitutively activated in clones stably transfected to express Q226L Ga13. Dominant negative forms of MEKK1 and MEKK4 were expressed stably in the clones harboring Q226L Ga13. Expression of dominant negative versions of either MEKK1 or MEKK4 effectively blocks both the activation of Jun N-terminal kinase as well as the formation of primitive endoderm. Depletion of MEKK1, -2, or -4 by antisense oligodeoxynucleotides suppressed signaling from Q226L Ga13 to JNK1 and primitive endoderm formation. We demonstrate that the signal linkage map from Ga13 activation to primitive endoderm formation in these stem cells requires activation at three levels of the mitogen-activated protein kinase cascade: MEKK1, -2, or -4 for MAP kinase kinase kinase; MKK4 and/or MEK1 for MAP kinase kinase; and JNK1 for MAP kinase.

The mitogen-activated protein (MAP)1 kinase network has emerged as an essential signaling component in development (1, 2). In a variety of model systems, members of the MAP kinase family play central roles. The activation of Erk1,2 is an obligate element in the ability of the morphogen retinoic acid (RA) to promote primitive endoderm (PE) formation in mouse F9 teratocarcinoma stem cells (3) and in the adiogenic conversion of mouse 3T3-L1 fibroblasts to yield mature adipocytes (4). Jun N-terminal kinase (JNK) has been implicated in differentiation of mouse 3T3-L1 fibroblasts to yield mature adipocytes (4). Jun N-terminal kinase (JNK) has been implicated in the development of early development, because three germ layers (endoderm, mesoderm, and ectoderm) can be derived from these mouse embryonal carcinoma cells in response to specific inducers and culture conditions (8). RA stimulates the loss of the embryonic marker SSEA-1 and positive staining by the TROMA antibody specific for the cytokeratin endo A, a hallmark for primitive endoderm.

The search to link RA action to activation of JNK revealed an important intermediary, the heterotrimeric G-protein Ga13. Expression of Ga13 rises sharply in P19 cells in response to RA as well as in early mouse embryos (8). In P19 cells, suppression of this rise in Ga13 by antisense oligodeoxynucleotides blocks the ability of RA to promote formation of PE (8). The concept that heterotrimeric G-proteins are critical elements of development has been bolstered by recent studies of developmental pathways in which the following α-subunits have been implicated: Gs, Go, Gq, Gi2, and G12/G13(2,9). One of the most powerful approaches to the study of G-protein α-subunits takes advantage of specific mutations that suppress the intrinsic GTPase activity, yielding an α-subunit that is constitutively activated. Herein we explored the upstream control of JNK activation in P19 clones that have been stably transfected to express the constitutively active (CA) Q226L mutant form of Ga13. Expression of Q226L Ga13 in P19 cells leads to robust activation of JNK and formation of PE in the absence of RA. At the level of MAP kinase kinase upstream of JNK, MEK1 and MKK4 (also known as JNKK1) are activated. At the level of MAP kinase kinase expression, Q226L Ga13 results in activation of MEKK1. Expression of the dominant negative form of either MEKK4 or MEKK1 blocks Q226L Ga13-induced differentiation. Treatment with oligodeoxynucleotides antisense to MEKK1, 2, or 4 blocks Q226L Ga13-induced JNK activation and PE formation. Thus, MEKK1, 2, and 4 act downstream of Ga13 and upstream of c-Jun N-terminal kinase in the pathway mediating differentiation of these stem cells to primitive endoderm.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—The P19 embryonal carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD). Both the stable transfectants and the wild-type clones were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified atmosphere of 6% CO2. P19 cells cultured as monolayers on tissue culture plates in the Dulbecco’s modified Eagle’s medium with 10% serum were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) in a humidified atmosphere of 6% CO2. P19 cells cultured as monolayers on tissue culture plates in the Dulbecco’s modified Eagle’s medium with 10% serum were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) in a humidified atmosphere of 6% CO2.

Plasmids and Transfections—pCDNA3 plasmids harboring no insert (i.e. empty vector), the wild-type, constitutively active, and dominant negative (DN) mutant forms of MEKK1 and MEKK4 were provided generously by Dr. Gary L. Johnson (Dept. of Pharmacology, University of Colorado Health Sciences Center, Denver, CO). The pCMV5 plasmid harboring the Q226L mutant form of Ga13 was obtained from Dr. Alfred Gilman (Pharmacology, University of Texas Southwestern Med-
ical School, Dallas, TX). The P19 cells were transfected with one or more plasmids using LipofectAMINE. Stably transfected P19 clones were selected in the presence of the neomycin analog G418 (400 µg/ml). The wild-type and mutant forms of the MEKK1 and MEKK4 had been epitope-tagged with the hemagglutinin antigen to follow expression of each independently of endogenous MEKKs.

**Immunoblotting**—Samples (10–50 µg of protein per lane) of total cell lysates were subjected to electrophoresis in SDS on 10% polyacrylamide gels. The resolved proteins were transferred electrophoretically to nitrocellulose blots. The blots were stained with primary antibodies and the immune complexes made visible using a second antibody coupled with isoperoxidase and developed using the ECL method. The antibodies were purchased from the following sources: JNK1, MEKK1, MEKK2, and MEKK7 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); MEK1 from Zymed Laboratories Inc. (South San Francisco, CA); MMK4 antibodies from Pharmogel Antibodies (San Diego, CA); phosphospecific antibodies to MEK1,2, MEK3,6, and MKK4 from Cell Signaling Technology (Beverly, MA).

**Immunoprecipitation and Activity Assays of MEKKs and JNK**—The immunoprecipitation reactions and solid-state assay of MEKKs and JNK were performed as detailed earlier (7), using the rGST-JNKK1 and rGST-c-Jun N-terminal fusion protein as substrates for the MEKKs and JNK activity assays, respectively. The vectors expressing the rGST-c-Jun and rGST-JNKK1 were provided generously by Dr. Roger Davis (HHMI, University of Massachusetts Medical Center, Worcester, MA). In some cases, P19 cells were treated with oligodeoxynucleotides antisense to MEKK1, 2, 3, and 4 as described earlier (8).

**Indirect Immunofluorescence Staining of SSEA-1 and TROMA**—The staining of the embryonic antigen SSEA-1 with the monoclonal antibody MC-80 and the endoderm-specific marker antigen cytokeratin stained, and subjected to analysis by epifluorescence microscopy as described previously. Because the differentiated cells often grow from monolayers to whorls of cells with multiple layers, the indirect immunofluorescence and phase contrast images may not appear to be in focus. This artifact is unavoidable under these conditions of differentiation.

**Data Analysis**—For all of the experiments reported, the data are compiled from at least three independent replicate experiments performed on separate cultures on separate occasions with highly reproducible results. The indirect immunofluorescence and phase contrast images are of representative fields of interest.

**RESULTS**

The mouse P19 embryonal carcinoma cells were stably transfected with an expression vector pCDNA3 either lacking an insert (empty vector) or harboring the cDNA for the constitutively active mutant form of Go13 with the Q226L substitution (Go13QL). The Q226L mutation reduces the intrinsic a-subunit GTPase activity responsible for the hydrolysis of GTP bound in the activated state of Go13. Analysis of the levels of Go13 mRNA in the stably transfected cells was performed by reverse transcription-polymerase chain reaction amplification. Expression of Go13 mRNA was increased dramatically in the clones harboring the Q226L Go13-containing expression vector (Fig. 1A). Amplification of the marker mRNA for GAPDH, used to evaluate loading consistency, was equivalent among the clones expressing Q226L Go13 or empty vector. Crude membranes prepared from P19 clones harboring empty vector or vector expressing the Q226L Go13 subunit were subjected to SDS-polyacrylamide gel electrophoresis, blotting to nitrocellulose, and staining with antibody against Go13 (Fig. 1B). Expression of Go13 Q226L as deduced from the increase in immunoreactive Go13 in blots of crude membranes of stably transfected clones was about 1.2- to 1.7-fold greater than the expression of the endogenous Go13 (Fig. 1C).

Treating P19 cells with RA induces activation of c-Jun N-terminal kinase and PE formation (7). Expression of Q226L Go13 mimics the activation of c-Jun N-terminal kinase by RA, as measured by the solid-state kinase assay (Fig. 2). Examined in a number of independent stably transfected clones, the increase in JNK activity ranged from 3.0- to 7.6-fold in clones expressing Q226L Go13, and the average increase in kinase activity over basal from more than five clones was 4.2-fold. The amount of JNK1 expression itself was monitored in the Q226L Go13-expressing clones and found to be the same as that observed for clones harboring the empty vector (Fig. 2) or for the wild-type cells (not shown).

The ability of Q226L Go13 to provoke JNK activation focused the analysis on the upstream control of kinase activation, commencing with a measurement of the expression and activity of members of the MAP kinase kinase family (Fig. 3). Expression of MEK1–4, 6, and MKK7 in the clones overexpressing Q226L Go13 was probed by immunoblotting. Expression of MKK2 only was modestly greater (10–15%) in the clones expressing the Q226L Go13 (Fig. 3A). The expression of MKK7 was similarly increased (not shown). The expression levels of MEK1, MEK3, and MKK4 were not changed (Fig. 3, A and B). MEK6 expression in F9 stem cells was not detected (not shown). Activation of MEKS occurs through phosphorylation of serine/threonine residues in MEK1 and 2 (Ser-217 and Ser-218).
expressing G
rect measurements of MEKK4 activity. The lack of suitable antibodies precluded similar di-
tion of MEKK1, the expression of MEKK1 itself was un-
immunoblotting and stained with an antibody to JNK1. JNK1 protein was
cipitants used for the solid-phase kinase assay were subjected to im-
dried, and subjected to autoradiography. Replicates of the immunopre-
tion products were analyzed on 10% SDS-PAGE. The gel was stained,
expressed GST-cJun was used as substrate. The phosphorylation reac-
tion products were analyzed on 10% SDS-PAGE. The gel was stained,
creased 1.5- to 2.0-fold in P19 cells expressing the G
crease 80 and 40%, respectively, in the clones expressing
JNK1 in the clones expressing the Q226L mutant of G
Q226L G
to a lesser extent MEK2 are constitutively activated similar to
expression of Q226L G
the constitutively activated Q226L G
levels of MEK activation are similar to reports in mouse
measured in these same stem cells in response to expression of
Expression of the dominant negative form of MEKK1 or MEKK4 likewise
abolished the ability of the Q226L Ga13 to activate JNK1 and
suppressed the basal activity of JNK1 below
expression and activation are shown (D). Asterisks denote values statis-
tically significant from the control with p ≤ 0.05.
Q226L Ga13, but it also suppressed the level of JNK1 below that observed in clones expressing the empty vector (Fig. 5). Expression of the dominant negative form of MEKK4 likewise abolished the ability of the Q226L Ga13 to activate JNK1 and suppressed the basal activity of JNK1 by 70%. These data demonstrate that activation of JNK by Ga13 Q226L mutant can be blocked by dominant negative forms of either MEKK1 or MEKK4.

Would the expression of DN-MEKK1 or DN-MEKK4, which blocks the ability of Q226L Ga13 to activate JNK, also block the ability of Q226L Ga13 to promote PE formation? P19 cells were stably co-transfected with expression vectors harboring Q226L Ga13 in the absence or presence of those harboring either DN-MEKK1 or DN-MEKK4. The clones were selected, propagated, and stained with either antibodies for the embry-
omic marker SSEA-1 or with TROMA, a monoclonal antibody that recognizes cytokeratin endo A, a PE-specific marker pro-
protein in early development (Fig. 6). Expression of Q226L Ga13 provoked not only the activation of JNK but also the loss of expression of the embryonic marker SSEA-1. The clones expressing Q226L Ga13 stain positively by TROMA, in the absence of the morphogen retinoic acid, demonstrating the ability of the constitutively active Gα13 to promote PE formation.

To further address the role of MEKK1–4 in JNK activation and PE formation, we examined the effects of oligodeoxynucleotides antisense to MEKK1, -2, -3, or -4 on P19 cells transiently transfected with an expression vector harboring Ga13Q226L (Fig. 7). Transient transfected P19 cells assayed at 48 h post-transfection displayed an increase in both JNK1 activity as well as in the PE marker stained with TROMA antibody (Fig. 7A), although less than that was obtained when the clones were allowed 96 h prior to sampling (which was not possible here). Cells transiently transfected with Ga13Q226L and treated with oligodeoxynucleotides (ODNs) antisense to MEKK1 (as MEKK1) displayed a loss of JNK1 activity and failed to form PE. Treatment with ODNs antisense to MEKK4 by the same approach also resulted in a reduction in JNK1 activity and failure to form PE. The treatment with antisense ODNs suppressed the expression of MEKK1–4, as determined by immunoblotting (Fig. 7B). Treating cells with ODNs antisense to MEKK2 displayed a suppression of JNK1 activity and a reduc-
that MEKK2 is capable of transducing Ga13Q226L signals into activation of JNK1 and PE formation, whereas MEKK3 may be involved in Ga13Q226L-stimulated PE formation in a pathway independent of JNK1 activation.

We explored whether the activation of JNK1 was obligate for PE formation in response to Ga13Q226L, as it is in response to PE formation caused by the morphogen retinoic acid. Cells transiently transfected to express Ga13Q226L were transfected concurrently with the dominant negative form of JNK1, JNK1(DN), and PE formation was assayed by immunoblotting of the TROMA1 antigen, which is a hallmark of PE. The expression of JNK1(DN) was assayed by immunoblotting with antibodies that stain endogenous and mutant Ga13 proteins alike. The results shown are representative of at least three separate experiments.

We examined also whether PE formation in response to expression of constitutively activated MEKK1 would be sensitive to the expression of the dominant negative JNK1. Expression of JNK1(DN) was confirmed by immunoblotting. The expression of Ga13Q226L provoked expression of the PE marker, and this Ga13Q226L-stimulated PE formation was blocked by co-expression of the JNK1(DN). We found that PE formation in response to Ga13Q226L or Ga13Q226L-stimulated PE formation in a pathway independent of JNK1 activation.

These data demonstrate that JNK1 activation is obligate for signaling from Ga13Q226L or MEKK1(CA) to PE formation.

**DISCUSSION**

The present studies explore the signaling upstream of JNK in a mammalian model of development, the pluripotent mouse P19 embryonal stem cell. A role for JNK in signaling primitive endoderm formation in response to the morphogen RA was first identified in the P19 stem cells (7,8) (Fig. 9). JNK has been implicated since in several models of development. In *Drosophila* dorsal closure, the basket gene product is an ortholog of JNK (18,19). The MAP kinase network has been implicated in *C. elegans* embryogenesis, although the jnk-1 gene product most
homologous to JNK appears to be obligate for coordinated locomotion (20). How the activity of JNK is activated in these systems remains to be fully elucidated. Expression of a constitutively active mutant form of Ga13 (Q226L) obviated the need for the morphogen and has been shown to promote robust PE formation (8). Expression of the Q226L Ga13 provokes persistent activation of JNK as well as formation of PE, enabling this group to detail signaling upstream of JNK in these mammalian stem cells.

At the level of MAP kinase kinase, it is clear that both MEK1 and MKK4 (which is also known as JNKK1) were persistently activated in clones expressing Q226L Ga13 (Fig. 9). MEK2 also displayed some activation, whereas MEK3 activity was unaffected by the expression of constitutively active Q226L Ga13. Upstream signaling of JNK in C. elegans requires the jnk-1 gene (20), whereas in Drosophila the hemipterus gene product is obligate (21). There appears to be a high degree of conservation for signaling pathways operating in development at the levels of the MAP kinase kinase and MAP kinase.

Direct analysis at the level of MEKK reveals the activation of MEKK1 in clones expressing Q226L Ga13, establishing a clear linkage between Ga13 and MAP kinase kinase alinals. Although direct analysis of MEKK4 was not possible, we employed the expression of dominant negative mutants of both MEKK1 and MEKK4 in an attempt to test if either of the DN-MEKKs could block constitutive signaling from Q226L Ga13 to JNK and PE formation. The results of the analysis demonstrate that expression of DN-MEKK1 blocks the activation of JNK1 as well as the ability of the Q226L Ga13 to promote PE formation. Expression of DN-MEKK4 also blocks signaling from Ga13 to JNK and PE formation. These observations were extended by studies in which cells were treated with ODNs antisense to MEKK1–4 individually. Treatment with ODNs antisense to MEKK1, 2, or 4 suppressed both JNK1 activation and PE formation in response to expression of Ga13Q226L. Depletion of MEKK3, in contrast, blocked PE formation in response to expression of Ga13Q226L, but failed to suppress JNK1 activation. Thus, signaling via MEKK1, 2, or 4 from Ga13Q226L to PE formation requires JNK1, although MEKK3 appears to play a role in Ga13Q226L-stimulated PE formation independent of JNK1 activation.

These studies reveal a central role for JNK1, and MEKK1/4 in the downstream signaling from Q226L Ga13 to the control of JNK and PE formation (Fig. 7). Orthologs of MKK4 and MEKK1/4 likely play a central role in the control of development in C. elegans (6, 22), Drosophila (23), and other models of development (1). Absence of the JNK and JNKK homologs, basket and hemipterus, in Drosophila leads to an absence in embryonic dorsal closure (5, 23–26). For mammalian P19 stem cells, inhibition of either JNK1 or MEKK4 by dominant negative versions leads to a block in the formation of primitive endoderm in response to either RA (17) or to the expression of Q226L Ga13 (present work). Expression of dominant negative JNK1 was shown to block PE formation in response to either Ga13Q226L or constitutively active MEKK1. Expression of the constitutively active form of JNK, in contrast, failed to stimulate PE formation (7), suggesting that additional signals, perhaps the result of activation of MAP kinase kinase or MAP kinase kinase kinase, are obligate for the PE formation to be initiated. In Drosophila, small molecular weight G-proteins have been implicated upstream of MAP kinase kinase activation in dorsal closure (21, 27). The fact that Ga13 can signal to members of the Rho family of G-proteins (28) provides an important lead for further study on the linkages between Ga13 and MEKKs in early vertebrate development (29).

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