c-Jun Amino-terminal Kinase Is Regulated by Go12/Go13 and Obligate for Differentiation of P19 Embryonal Carcinoma Cells by Retinoic Acid*

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Retinoic acid induces P19 mouse embryonal carcinoma cells to differentiate to endoderm and increases expression of the heterotrimeric G-protein subunits Go12 and Go13. Retinoic acid was found to induce differentiation and sustained activation of c-Jun amino-terminal kinase. Much like retinoic acid, expression of constitutively active forms of Go12 and Go13 induced differentiation and constitutive activation of c-Jun amino-terminal kinase. Expression of the dominant negative form of c-Jun amino-terminal kinase 1 blocked both the activation of c-Jun amino-terminal kinase and the induction of endodermal differentiation in the presence of retinoic acid. These data implicate c-Jun amino-terminal kinase as a downstream element of activation of Go12 or Go13 obligate for retinoic acid-induced differentiation.

The role of heterotrimeric guanine nucleotide binding proteins (G-proteins) in cell differentiation and development has been shown in several systems (1–4). Adipogenesis of NIH 3T3-L1 cells in response to inducers such as insulin or dexamethasone plus methylisobutylxanthine is accompanied by a sharp decline in Go subunit (5). Both suppression of Go subunit by antisense oligodeoxynucleotides and overexpression of the constitutively active mutant form of Go promote adipogenesis in the absence of classical inducers (5). Differentiation of F9 embryonic stem cells to endodermal cells provokes a sharp reduction of Go2 (2), a de-repression of phospholipase C, and activation of protein kinase C and mitogen-activated protein (MAP) kinase, especially via ERK1,2 (6). The expression of constitutively active forms of Go2 and Go13 can induce neuronal differentiation of PC 12 cells (7), via activation of c-Jun amino-terminal kinase but not via ERK1,2.

The extracellular signal-regulated kinase (ERK), stress-activated protein kinase (SAPK/JNK), and mammalian homolog of the yeast-osmosensing ERK HOG1 (p38 MAPK) are conserved members of a MAP kinase cascade for regulation of targets such as transcription factors in response to growth factors or environmental stresses, such as ultraviolet light, and protein synthesis inhibitors (8–16). ERK appears to play a major role in provoking cell proliferation and differentiation (17–19), whereas JNK mediates stress responses and some forms of apoptosis (20–23). JNK has been implicated also in the induction of differentiation (7, 24) and oncogenesis (25). The ability of overexpression of c-Jun, which is a target for JNK, to stimulate differentiation of P19 embryonal carcinoma cells to a mixed endoderm/mesoderm population supports a role of JNK in differentiation (26).

P19 embryonal carcinoma (P19) cells have been used as a model system for murine pre-implantation development (27, 28). These pluripotent cells have the ability to differentiate into derivatives of three germ layers, endoderm, mesoderm, and ectoderm, upon different inducer stimulation (29, 30). Monolayer cultures of P19 cells challenged with 50 nM retinoic acid (RA) differentiate to endodermal-like cells (31).

In the current study we show that JNK activity is increased during RA-induced differentiation of P19 cells and the progression of P19 cell differentiation by Go12 and Go13 is mediated by JNK, but not ERK or p38 MAPK. Expression of the dominant negative form of JNK1 is shown to block RA-induced differentiation of embryonal cells to endoderm, establishing an obligate role of JNK in a model of early mouse embryogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—The P19 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) in 6% CO2 humidified chamber. To induce endodermal differentiation, P19 cells were cultured as monolayers on tissue culture plates in growth media supplemented with 10 nM RA (all-trans; Sigma) for 2–5 days.

Plasmids and Transfection—The antisense sequences, 5'-AGCT-TCAGCCGGGCGCCTCGGGCCTGGGCCCCGGCGGCGGCTAT-3' and 5'-AGCTTTCGCGCGCCCTGGGCCCCGTGGGCCCCGGTGGGGTGGGGTGCTTT-3' derived from the cDNA sequences of mouse Go12 and Go13 (32), respectively, were engineered into the HindIII/EcoRI sites of the pcDNA3 retroviral vector using standard recombinant DNA techniques (33). Plasmids (34) harboring the constitutively activated form of Go12 (pcDNA3-Go12Q226L) or of Go13 (pcDNA3-Go13Q226L) were obtained from Dr. Gary L. Johnson (National Jewish Center for Immunology, Denver, CO). The plasmid containing the cDNA for the Flag epitope-tagged, dominant negative form of JNK1 replaces the dual phosphorylation sites Thr-Pro-Tyr with Ala-Pro-Phe (pCMV5-JNK1APF) and was employed to probe the role of JNK in P19 cell differentiation. P19 cells were transfected with plasmids using Lipofectin® (Life Technologies, Inc.), according to the manufacturer’s protocol. For pCMV5-JNK1-APF, the pcW1-neo plasmid was co-transfected to provide a selectable marker. Positive transfectants were selected using G418 (400 mg/ml,
Preparation of Total Cell Lysate—For immunoprecipitations and FPLC analysis, P19 cells were washed with phosphate-buffered saline (pH 7.2) and lysed. Lysis buffer contains 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH2PO4, 10 mM sodium molybdate, 20 mM Tris-HCl (pH 7.4), 5 mg/ml aprotinin, 5 mg/ml leupeptin, 6.0 mM dithiothreitol, 2 mM sodium orthovanadate, 200 mM phenylmethylsulfonyl fluoride, 0.1% sodium dodecyl sulfate, and 1% Triton X-100. After 20 min at 4 °C with constant rotation the cell lysate was subjected to centrifugation at 14,000 \times g for 15 min, and the resultant supernatant was transferred to a fresh tube. Protein content was measured by the Lowry method (35), using bovine serum albumin as the standard.

Immuno blotting Analysis—Aliquots of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred electrophoretically from the gel to nitrocellulose membrane. Antibodies to the following antigens employed in these studies were obtained from the indicated sources: JNK 1, Santa Cruz Biotechnology, Santa Cruz, CA; ERK1 and 2, Zymed Laboratories Inc., San Francisco; p38 MAPK, Upstate Biotechnology, Lake Placid, NY, Go12, Dr. Gunther Schultz, Freie University, Berlin, Germany and Santa Cruz Biotechnology, Santa Cruz, CA; and Go13, Dr. Gunther Schultz, Freie University, Berlin, Germany and Santa Cruz Biotechnology, Santa Cruz, CA, or a rabbit polyclonal antiserum obtained by immunization with a synthetic C-terminal dodecapeptide of Go13. The immune complexes formed were made visible by using alkaline phosphatase-linked goat anti-rabbit IgG (Life Technologies, Inc.) or rabbit anti-mouse IgG (Life Technologies, Inc.), or rabbit anti-goat IgG (Sigma).

Immunoprecipitation of JNK and JNK Assay—An aliquot (250 μg to 1 mg of protein) of the clarified lysate was mixed with the JNK1-specific antibody and resuspended to 1.0 ml in the same cell lysis buffer. After 2 h at 4 °C with constant rotation, 20–40 μl of Protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the incubation was continued for an additional 1.5 h. The immunoprecipitates were pelleted by centrifugation at 14,000 \times g for 5 min. The pellets were washed twice with same lysis buffer and then washed twice with JNK assay buffer (20 mM HEPES (pH 7.5), 20 mM MgCl2, 0.1 mM Na3VO4, 2 mM dithiothreitol, 20 mM β-glycerophosphate). Immunoprecipitates were resuspended in a final volume of 40 μl of the JNK assay buffer. A 20-μl aliquot was used for Western blot analysis and the other 20 μl was used for JNK assay. Aliquots of immunoprecipitates were mixed with 2 μg of GST-fusion proteins encoding for the N-terminal region of Jun N-1 (1–79) (36) and placed on ice. Reaction was started by addition of [γ-32P]ATP (10 μCi/tube, 100 μM final concentration) and incubated for 15 min at 30 °C. Reaction was stopped by addition of 6 μl of 4 × Laemmli buffer (200 mM Tris-HCl (pH 6.8), 0.4 mM dithiothreitol, 8% SDS, 0.08% bromophenol blue, and 40% glycerol). After separation of proteins by 10% SDS-PAGE and staining with Coomassie Brilliant Blue R250 and autoradiography, the bands for GST-Jun were excised and the incorporated 32P was quantified by liquid scintillation counting.

Source 15Q FPLC—Undifferentiated and differentiated P19 cells were harvested and lysed. An aliquot of sample (2.5 mg of protein) was resuspended in 1 ml of lysis buffer and fractionated by chromatography on a Mono Q HR 5/5 FPLC column (Pharmacia Biotech Inc., Uppsala, Sweden). The column was washed with 8 column volumes of buffer A (70 mM β-glycerophosphate (pH 7.2), 0.1 mM Na3VO4, 2 mM MgCl2, 1 mM EGTA, and 1 mM dithiothreitol), and the bound proteins were eluted with a 12.5-mL linear gradient of NaCl (0–400 mM) in buffer A. Twenty-five fractions (0.5 ml) were collected at a flow rate of 1 ml/min.

ERK and p38 Protein Kinase Assays—A 15-μl aliquot of FPLC fraction was mixed with 5 μl of 4 × kinase assay buffer (100 mM β-glycerophosphate, 160 mM HEPES (pH 7.2), 0.2 mM Na3VO4, 40 mM MgCl2, 100 μg/ml protein kinase A inhibitor peptide, 2 mM EGTA) and 2 μg of either MBP (Sigma) or GST-ATF2(1–109) (36) used as substrates. Each reaction was started by addition of [γ-32P]ATP (10 μCi/tube, 100 μM final concentration) and incubated for 15 min at 30 °C. The reactions were terminated by addition of 6 μl of 4 × Laemmli buffer. After separation of proteins by SDS-PAGE on 12% acrylamide gels, staining of the resolved proteins with Coomassie Brilliant Blue R250, and autoradiography, the bands for either MBP or GST-ATF2(1–109) were excised, and the incorporated 32P into these substrates was quantified by liquid scintillation counting.

Indirect Immunofluorescent Methods and Antibodies—Cells were grown in Lab-Tek chamber slides (Nunc, Rochester, NY) and were fixed for 10 min with 3% paraformaldehyde. After fixation, slides were rinsed three times with modified Shield’s media/Pipes (modified Shield’s medium: 18 mM MgSO4, 5 mM CaCl2, 40 mM KCl, 24 mM NaCl, 5 mM Pipes (pH 6.8), 0.5% Triton X-100, 0.5% Nonidet P-40). A monoclonal antibody MC-480 (SSEA-1) that reacts with a stem-cell surface antigen marker (37) was used to identify the embryonal-stage cells. MC-480 was obtained from the NIH Developmental Studies Hybridoma Bank (Balti-
more, MD). The fixed cells were incubated with primary antibody for 30 min at 37 °C and then washed three times with modified Shield's media/Pipes buffer. Fluorescein-conjugated goat anti-mouse (Kirkgaard & Perry Laboratories, Gaithersburg, MD) was added and incubated for 30 min at 37 °C. The slides were washed then three times with blotting buffer (500 mM NaCl, 10 mM KPO4 (pH 7.5), 0.1% Triton X-100, 0.02% SDS). The cells were examined by phase contrast and epifluorescence microscopy on a Zeiss Axiphot microscope and photographed on Kodak-64T or Kodak-TMax 400 film.

RESULTS AND DISCUSSION

The P19 cells differentiate to neuroectoderm-like phenotypes (29, 30) when allowed to aggregate in the presence of 100 nM retinoic acid. In the absence of aggregation, P19 cells are induced to differentiate by RA but only to the endoderm-like phenotype (29, Fig. 1), providing a useful model for study of this process. P19 embryonic cells stain positive in indirect immunofluorescence study of cells probed with antibody to the embryonic marker SSEA-1 (Fig. 1, panel B). The cells induced morphologically to endoderm by RA no longer stain positive with the same antibody, displaying a loss in the embryonic marker antigen SSEA-1 (Fig. 1, panel D). Endodermal cells stain positive with TROMA-1, a monoclonal antibody to the endoderm-specific marker cytokeratin-1 (37, 38). Untreated P19 cells stain negative for TROMA-1 (Fig. 1, panel F), reflecting their embryonal character, whereas RA-treated P19 cells stain prominently for TROMA-1 (Fig. 1, panel H), a hallmark for endodermal development (37, 38). The absence of epifluorescence signals from the RA-treated cells stained with SSEA-1, from the untreated P19 cells stained with TROMA-1, and from the controls performed without primary antibody (not shown) provide ample evidence for the specificity of the staining in defining endoderm versus embryonal character (Fig. 1). Since heterotrimeric G-proteins have been implicated in control of P19 cell differentiation (39) and Ga12 and Ga13 are known to mediate the activation of mitogen-activated protein kinases, especially c-Jun amino-terminal kinase (16, 40), we explored the role of MAP kinases in P19 cell differentiation, with an emphasis on Ga12 and Ga13.

To explore the role of MAP kinases in P19 cell differentiation, cells were treated with retinoic acid (100 nM) for 3 days to achieve full differentiation to endodermal phenotype and a cell extract prepared and fractionated by FPLC on a Source Q column (Fig. 2A). Immunoblotting of samples from the fractions revealed ERK1,2 in fractions 9–11 and p38 in fractions 14–19 of extracts from untreated P19 cells. MAP kinase activity was measured using myelin basic protein (MBP), which is a substrate for both p38 and ERK1,2. The profile of activities coincides with the appearance of ERK1,2 and p38 kinases in the immunoblots, shown as insets (Fig. 2, panel A). When MAP kinase activity was measured in fractions from an FPLC of RA-treated, endodermal cells, a modest but highly reproducible (n = 3) decline in ERK1,2 activity was observed in each profile (Fig. 2, panel A). MAP kinase activity in the region of the profile in which p38 was found increased slightly in the extracts from the RA-treated, endodermal P19 cells in each of three separate analyses (Fig. 2, panel A). The status of p38 kinase activity in the FPLC fractions from untreated and RA-treated P19 cells was measured further, using GST-ATF2 fusion protein, as ATF2 is a specific substrate for p38 and not ERK1,2.
3) on day 2 and 7.0

Anisomycin precipitated a return to baseline of the JNK activity by more than 3-fold within 2 h of stimulation. The activities show a modest decline.

Unlike the modest changes observed in the activity of p38 or ERK1,2 in the RA-treated cells, c-Jun amino-terminal kinase activity (JNK) displays marked activation upon treatment of the cells with RA (Fig. 2C). JNK activity in the solid-state assay using GST-Jun fusion protein increases from 2.7-fold on day 2 to 7.8-fold on day 3 following induction of differentiation in the absence of RA treatment. Based upon multiple experiments, the increase in JNK activity (fold over basal) was found to be 

\[ 4.3 \pm 0.6 \ (n = 3) \] on day 2 and 
\[ 7.0 \pm 0.2 \ (n = 4) \] on day 3 following treatment with RA. The amount of the JNK itself, in contrast, was found to endoderm by RA, p38 activities increase little, and ERK1,2 displayed activation of JNK activity ranging from 1.9- to 2.7-fold over basal levels. P19 cells expressing Q226L Go_{12} or Q229L Go_{13} that are constitutively active were probed with respect to JNK activation, in the absence of RA treatment. Expression of either Q226L Go_{12} or Q229L Go_{13} in P19 cells resulted in the activation of JNK activity in the absence of RA treatment. Cells expressing Q226L Go_{12} or Q229L Go_{13} in P19 cells resulted in the activation of JNK activity ranging from 2.4- to 3.8-fold over basal (Fig. 4). In the absence of RA treatment, P19 cells expressing Q229L Go_{13} have been shown to differentiate to the endodermal phenotype (39). Expression of Q226L Go_{12} induces the differentiation from embryonal phenotype and loss of positive staining for the SSEA-1 embryonic-specific marker but not to endodermal-like phenotype (39). These data are consistent with a role for Go_{12} and Go_{13} controlling aspects of differentiation induced by morphogens (38) but go further to demonstrate a potential role for activation of the MAP kinase regulatory network, and specifically JNK, by RA.

The linkage between expression of Go_{12} and Go_{13} and JNK activity in differentiating P19 cells was approached from the

**Fig. 4.** Expression of either constitutively active Go_{12} (Q229L) or Go_{13} (Q229L) activates c-Jun amino-terminal kinase in P19 embryonal carcinoma cells. JNK was immunoprecipitated with JNK1 antibody from 1.0 mg of lysate prepared from stable clones harboring empty vector alone (pCDNA3) or expressing constitutively active mutant forms of either Q229L Go_{12} (Go_{12}QL) or Q229L Go_{13} (Go_{13}QL). JNK kinase assays were performed using GST-Jun as the substrate (GST-Jun, top). The levels of JNK were equivalent among all of the experimental groups. These experiments were replicated twice, each with at least two separate clones, with identical results.

**Fig. 3.** Retinoic acid, but not anisomycin, stimulates persistent activation of c-Jun amino-terminal kinase. Panel A, P19 cells were incubated without (lane 1) and with 100 ng/ml anisomycin for 2 h and the cells assayed either immediately (lane 2) or 1 day (lane 3) following drug removal (lane 3). JNK activity (top) was assayed as described in the legend to Fig. 4. Blots stained with JNK1 antibody after immunoprecipitation (IP) (bottom) show the presence of equivalent amounts of JNK among the experimental groups. Panel B, P19 cells were incubated without RA (lane 1) and either 100 nM RA for 5 days (lane 2) or 100 nM RA for 2 days (2d) followed by 3 days (3d) without RA (lane 3) and JNK activity measured (top). Immunoblotting of the immune precipitates stained with JNK1 antibody (bottom) show the presence of equivalent amounts of JNK among the experimental groups. The data shown are representative of three experiments, replicated on separate occasions.
FIG. 5. Suppression of either Ga12 or Ga13 by antisense RNA attenuates the activation of c-Jun amino-terminal kinase in P19 embryonal carcinoma cells. JNK was immunoprecipitated with JNK1 antibody from 1.0 mg of lysate prepared from stable clones harboring empty vector alone (pLNCX) or clones expressing RNA antisense to Ga12 (AS Ga12) or to Ga13 (AS Ga13). JNK kinase assays were performed using GST-Jun as the substrate (GST-Jun, top). The products of the kinase reactions were resolved by SDS-PAGE on 10% gels, detected by autoradiography, and quantified by scintillation counting. Portions of immunoprecipitates were subjected to SDS-PAGE, the resolved proteins transferred to nitrocellulose membranes, and the resultant blots probed with antibody specific for JNK1 (IP/Blot, JNK1, bottom). The levels of JNK were equivalent among all of the experimental groups. These experiments were replicated twice, with two separate clones for each antisense construct, with identical results.

The absence and presence of RA (Fig. 6) expressed a dominant negative mutant form of JNK1 (JNK1 activation and induction of differentiation by RA, we stably transfected either with empty vector (pCMV5) alone or with pCMV5-JNK1APF (JNK1 APF) vector harboring the dominant negative form of JNK1 were incubated in the absence or presence of 200 ng/ml of anisomycin for 2 h. JNK was immunoprecipitated with JNK1 antibody from cell lysates (0.25 mg of protein) of vector alone and pCMV5-JNK1/APF transfected cells. Kinase assays were performed using the substrate GST-Jun. The product of phosphorylation was resolved by SDS-PAGE on 10% gels, detected by autoradiography, and quantified by scintillation counting. JNK activity is expressed as the fold induction of JNK activity in response to the induction of JNK activity upon RA treatment.

To test further the linkages among Ga12 and Ga13, JNK activation and induction of differentiation by RA, we stably transfected Ga12 and Ga13 clones in stable transfectants of P19 cells using the pLNCX vector (39). Suppression of Ga13 was 50–70% in clones expressing RNA antisense to Ga12 (39), whereas induction of JNK activity upon RA treatment in those clones was attenuated significantly in comparison to clones harboring the empty vector (Fig. 5) or to wild-type cells (Fig. 2C). The same basic observations were true for the clones in which RNA antisense to Ga13 suppressed Ga13 levels 60–80%, and induction of JNK activity upon RA treatment was found to be reduced by approximately half. Thus, expression of constitutively active forms of Ga12 and Ga13 results in constitutively elevated basal levels of JNK activity, whereas suppression of Ga12 and Ga13 results in constitutively attenuated induction of JNK activity upon RA treatment.

To test the ability of the dominant negative to block endogenous JNK1 activity, we probed activity of JNK1 following anisomycin activation in cells stably expressing either pCMV vector alone or pCMV vector harboring JNK1 (APF). Upon treatment with 200 ng/ml anisomycin, control cells displayed a 15-fold activation of JNK over basal levels, whereas cells expressing the dominant negative showed a 4-fold activation (Fig. 7B). These data strongly suggest that induction of JNK activity showed a causal effect for differentiation rather than result of differentiation.

The capacity to abolish the activation of JNK in response to RA by expressing the dominant negative form of JNK1 provided the opportunity to address directly if the activation of JNK is critical to the ability of RA to induce differentiation of the embryonal P19 cells. Clones harboring the empty vector (pCMV5) as well as those expressing the dominant negative version of JNK1 were both treated with RA for 3 days and then

FIG. 6. Expression of dominant negative c-Jun amino-terminal kinase blocks activation of c-Jun amino-terminal kinase in response to retinoic acid and attenuates activation by anisomycin. Panel A, P19 clones stably transfected either with empty vector (pCMV5) alone or with pCMV5-JNK1APF (JNK1 APF) vector harboring the dominant negative form of JNK1 were incubated in the absence or presence of 100 nM RA for 3 days. JNK was immunoprecipitated with JNK1 antibody from cell lysates (0.25 mg of protein) of vector alone and pCMV5-JNK1/APF transected cells. Kinase assays were performed using the substrate GST-Jun. The product of phosphorylation was resolved by SDS-PAGE on 10% gels, detected by autoradiography, and quantified by scintillation counting. JNK activity is expressed as the fold induction of JNK activity in response to the induction by RA. Aliquots of immunoprecipitated JNK were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and the blots stained with antibody specific for JNK1 (inset). The immunoblotting demonstrates equivalence of JNK1 among the experimental groups. Panel B, P19 clones stably transfected either with empty vector (pCMV5) alone or with pCMV5-JNK1APF (JNK1 APF) vector harboring the dominant negative form of JNK1 were incubated in the absence or presence of 200 ng/ml of anisomycin for 2 h. JNK was immunoprecipitated with JNK1 antibody from cell lysates (0.25 mg of protein) of vector alone and pCMV5-JNK1/APF transected cells. Kinase assays were performed using the substrate GST-Jun. The product of phosphorylation was resolved by SDS-PAGE on 10% gels, detected by autoradiography, and quantified by scintillation counting. JNK activity is expressed as the fold induction of JNK activity in response to the induction by RA. Aliquots of immunoprecipitated JNK were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and the blots stained with antibody specific for JNK1 (inset). The immunoblotting demonstrates equivalence of JNK1 among the experimental groups. These data are from experiments replicated at least twice with identical results.

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stained with antibody to the embryonic-specific marker SSEA-1 (Fig. 7). When treated with RA, clones harboring the empty vector differentiated to the endodermal phenotype just as wild-type P19 cells (Fig. 1), displaying negative staining for the embryonic marker antigen, SSEA-1 (Fig. 7). Clones expressing the dominant negative form of JNK1, in contrast, stain positive for the embryonic marker in the absence or presence of RA. These data demonstrate an obligate role for JNK1 activation in the differentiation of P19 cells to endoderm stimulated by RA.

P19 embryonal carcinoma cells are an attractive model for the study of differentiation, displaying a capacity to differentiate to endoderm, neuroectoderm, and beating cardiac myocytes under the appropriate culture conditions (29, 30). The P19 cell line was adopted for the study of the role of heterotrimeric G-protein subunits G<sub>12</sub> and G<sub>13</sub> as well as MAP kinases in the differentiation of these embryonal cells to endoderm in response to RA. G<sub>12</sub> and G<sub>13</sub> are expressed in P19 cells, and their levels of expression are increased in response to RA (39). Moreover, expression of the constitutively active mutants of G<sub>12</sub> and G<sub>13</sub> provoke differentiation from the embryonal phenotype in the absence of RA (39). The role of heterotrimeric G-proteins in the regulation of the MAP kinase regulatory network has been appreciated recently, and the current study illuminates the role of G<sub>12</sub> and G<sub>13</sub> in differentiation mediated via specific activation of JNK but neither ERK1,2 nor p38. The JNK pathway is required for embryonic viability (42). Transgenic mice in which the gene for MKK4, the upstream activator of JNK, has been interrupted die prior to day 11 of embryonic development (42). This lack of viability is consistent with a role of JNK in mammalian development, perhaps differentiation (42). In Drosophila, the JNK pathway is required for embryonic development (43–45). Embryonic death occurs because of a failure of the migration of the dorsal epithelial cells required for dorsal closure. The early nature of this defect precludes analysis of the role of JNK in differentiation in this system (43–45). The most compelling evidence for the role of JNK1 in P19 embryonal cell differentiation was the ability of the dominant negative mutant of JNK1 to block endodermal differentiation in response to RA. Perhaps these heterotrimeric G-proteins activate JNK via known downstream elements (46) or perhaps through a tyrosine kinase that is regulated directly by G-proteins, as recently reported in an avian lymphocyte cell line (47).

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