Disassociation of MAPK Activation and c-Fos Expression in F9 Embryonic Carcinoma Cells following Retinoic Acid-induced Endoderm Differentiation*

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Retinoic acid induces cell differentiation and suppresses cell growth in a wide spectrum of cell lines, and down-regulation of activator protein-1 activity by retinoic acid contributes to these effects. In embryonic stem cell-like F9 teratocarcinoma cells, which are widely used to study retinoic acid actions on gene regulation and early embryonic differentiation, retinoic acid treatment for 4 days resulted in suppression of cell growth and differentiation into primitive and then visceral endoderm-like cells, accompanied by a suppression of serum-induced c-Fos expression. The MAPK (ERK) pathway was involved in mitogenic signaling in F9 cells stimulated with serum. Surprisingly, although c-Fos expression was reduced, the MAPK activity was not decreased by retinoic acid treatment. We found that retinoic acid treatment inhibited the phosphorylation of Elk-1, a target of activated MAPK required for c-Fos transcription. In F9 cells, the MAPK/MEK inhibitor PD98059 suppressed Elk-1 phosphorylation and c-Fos expression, indicating that MAPK activity is required for Elk-1 phosphorylation/activation. Phosphoprotein phosphatase 2B (calcineurin), the major phosphatase for activated Elk-1, is not the target in the disassociation of MAPK activation and c-Fos expression since its inhibition by cyclosporin A or activation by ionomycin had no significant effects on serum-stimulated c-Fos expression and Elk-1 phosphorylation. Thus, we conclude that retinoic acid treatment to induce F9 cell differentiation uncouples Ras/MAPK activation from c-Fos expression by reduction of Elk-1 phosphorylation through a mechanism not involving the activation of phosphoprotein phosphatase 2B.

Retinoic acid is thought to be a master regulator in mammalian development (1, 2). A gradient of retinoic acid is found in the developing embryo (1, 2), and retinoic acid plays a role in anterior-posterior determination, cell lineage induction, cell differentiation, organogenesis, and cell positioning (3–5). In adult tissues, retinoic acid also functions to maintain cell differentiation and is required for organ regeneration (6, 7). In tissue culture, retinoic acid induces cell differentiation and suppresses cell growth in a wide spectrum of cell lines (4, 5). The multipotent F9 embryonic carcinoma cells are often used as a model to investigate the mechanism of retinoic acid in cell differentiation and cell growth control and the biochemical basis of early embryonic development (8–11). Upon exposure to retinoic acid, F9 cells differentiate into primitive endoderm-like cells, accompanied by a reduction of cell growth (10, 11). The primitive endoderm cells, which can be further differentiated into visceral and parietal endoderm cells, are organized into a monolayer by a sheet of basement membrane in the early embryos (12, 13). The differentiated F9 cells express endoderm markers such as GATA-4 (14), GATA-6 (15, 16), and Dab2‡ (17, 18) and basement membrane components including collagen IV and laminin (10, 19, 20), thus resembling embryonic endoderm cells in many biochemical properties.

The Ras/MAPK pathway is involved in the induction of primitive endoderm differentiation of F9 cells (21), and suppression of the pathway promotes embryonic stem cells for self-renewal (22). Up-regulation of AP-1 activity by retinoic acid contributes to cell differentiation (23). c-Fos, a component of the AP-1 complex, is expressed during the development of the early embryos (24). An early study suggested that c-Fos expression may be required for differentiation of F9 cells (25). However, additional investigations have observed that c-Fos expression is not increased during F9 cell differentiation (23, 24, 26) and is not essential or sufficient for F9 cell differentiation (27–30).

The Ras/MAPK pathway is conserved in yeast, Caenorhabditis elegans, Drosophila, and mammals and functions in development, cell regulation, growth, and differentiation (31). In mammalian cells, the Ras/MAPK pathway mediates cell signaling of many growth factor receptor tyrosine kinases. Through adapters, GDP/GTP exchange to activate Ras, and a kinase cascade, the extracellular signal causes the phosphorylation/activation of cellular MAPK (ERK) (32–34). One established target for MAPK is Elk-1, a transcription factor required for transactivation of c-Fos (35–38). Subsequently, the AP-1 complex, of which c-Fos is a component, is assembled for gene transcription that mediates the biological response (38–40). c-Fos was first identified as a cellular counterpart of a viral oncogene capable of cell transformation (41). An immediate-early gene whose transcription is activated by serum and

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1 The abbreviations used are: Dab2, Disabled-2; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; MTI, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; PP2B, phosphoprotein phosphatase 2B; KSR, kinase suppressor of Ras; Grb2, growth factor receptor-binding protein-2; Gab2, Gab2-associated binder-2; DOS, Daughter of Sevenless.

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growth factors (42, 43), its expression is a key switch in cell regulation (40, 44). c-Fos, together with c-Jun, forms the AP-1 transcriptional complex for the transcription of many genes important for cell cycle progression, including cyclin D1 (40, 44, 45). Many studies have suggested that down-regulation of c-Fos expression interferes with the proliferation of tumor cells in vitro (45–47). The physiological importance of c-Fos expression for proliferation and transformation is not as certain. Although gene knockout studies indicate that c-Fos is dispensable for cell proliferation and mouse development (48), it contributes to and is essential for the malignant growth of solid tumor cells (49).

In differentiated cells, retinoic acid can suppress c-Fos expression (50–52), leading to growth reduction and apoptosis, suggesting that repression of c-Fos expression may be part of the differentiating activity of retinoic acid in somatic cells. We found that retinoic acid treatment for 4 days resulted in suppression of serum-induced c-Fos expression in F9 cells, and this reduction occurred during differentiation of F9 to endoderm-like cells. Surprisingly, the Ras/MAPK activity was not affected by retinoic acid treatment, although the phosphorylation of Elk-1 was inhibited. Thus, we conclude that retinoic acid treatment uncouples Ras/MAPK activation from c-Fos expression by reduction of Elk-1 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—All-trans-retinoic acid and dibutyryl cAMP were purchased from Sigma. Kinase inhibitors PD98059 and SB202190, cyclosporin A, and ionomycin were purchased from Calbiochem. Tissue culture supplies were obtained from Fisher. Dulbecco’s modified Eagle’s medium and fetal bovine serum (FBS) were purchased from Mediatech (Herndon, VA). The ECL Super-Signal West Dura Extended Duration Substrate immunodetection kit was purchased from Pierce. All other general chemicals and supplies, including Me2SO, ethanol, ispropyl alcohol, and agarose, were from Sigma or Fisher and were reagent grade or higher.

**Cell Culture**—F9 mouse teratocarcinoma cells were purchased from American Type Culture Collection. The cells were cultured on tissue culture plates coated with 0.1% gelatin in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS and antibiotic/antimycotic solution. Cells were maintained at 37 °C and 5% CO2 in a humidified tissue culture incubator.

All-trans-retinoic acid was dissolved in Me2SO to a stock concentration of 0.1 mM. Dibutyryl cAMP was dissolved in water to make a 50 mM stock. PD98059 and SB202190 were dissolved in Me2SO to make 50 and 20 mM stocks, respectively. These reagents were aliquoted, stored at −20 °C, and kept from light exposure until used. For serum stimulation experiments, the cells were first cultured for 18 h without serum in 1% bovine serum albumin or in low serum (0.5% FBS) and then were stimulated with 15% FBS for various times.

**MTT Assay**—Cell growth and cell numbers were estimated using the MTT assay (Promega) according to the manufacturer’s directions. Briefly, the cells were cultured in 96-well gelatin-coated plates (~103 cells/well) under the specified experimental conditions with the addition of tested compounds. All experiments were performed in triplicate. The medium was changed every 2 days. At the end of the specified incubation period, the MTT reagent (15 μl) was added to cells and incubated for 2–4 h. The reactions were terminated by the addition of stop/solubilization solution (100 μl). Cell numbers were assessed spectrophotometrically at 570 nm by determining the conversion of tetrazolium salt to a colored formazan product. In our experience, the results from the MTT assay are consistent with cell number determined by counting.

**Antibodies and Western Blot Analysis**—Polyclonal anti-Dab2 antibodies were characterized as previously described (53) and used for immunoprecipitation. Monoclonal anti-Dab2 (p96) antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-c-Fos antibodies were from Santa Cruz Biotechnology. Anti-actin antibodies were from Sigma. Anti-ERK1/2 and anti-phospho-ERK1/2 antibodies were from Cell Signaling Technology. Immunoblotting was performed according to standard procedures as described previously (53) using a chemiluminescence system (Pierce). After confirmation of antibody selectivity, two or more antibodies were in some cases used simultaneously in an incubation to detect various molecular mass proteins by Western blot analysis.

**RESULTS**

**MAPK Pathway Mediates Mitogenic Signaling in F9 Mouse Teratocarcinoma Cells**—F9, an embryonic stem cell-like teratocarcinoma cell line, can be differentiated into primitive and visceral endoderm by treatment with retinoic acid (9–11) and is often used in studies of early embryonic development and retinoic acid regulation (10). Retinoic acid induced F9 cell differentiation, accompanied by cell growth suppression (Fig. 1), which is mediated by retinoic acid receptor-β (10). Similar to many somatic cells, proliferation of F9 cells is serum-dependent. The MAPK pathway is involved in this mitogenic signaling since the MAPK/MEK inhibitor PD98059 inhibited serum-stimulated cell growth (Fig. 1). Retinoic acid treatment also inhibited F9 cell growth, but to a smaller degree than PD98059. In a representative example of four experiments, cell number following growth in the absence of serum was 16% of control.
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One consequence of MAPK activation is the expression of immediate-early genes such as c-fos (38, 42, 43). Retinoic acid treatment for 4 days suppressed serum-stimulated c-Fos expression in F9 cells, as indicated by Western blotting (Fig. 2), consistent with previous reports (23, 24, 26–30). In serum-deprived F9 cells, serum stimulation resulted in elevated c-Fos expression after 30 min and maximal expression by 90 min. In retinoic acid-treated cells, however, c-Fos expression was greatly suppressed (Fig. 2). The decrease in c-Fos protein in retinoic acid-treated cells correlates with a reduced level of c-Fos mRNA detected by Northern blotting (data not shown). When the period of retinoic acid exposure was less than 1 day, retinoic acid had no effect on c-Fos expression in F9 cells (data not shown), and the inhibition of cell growth could be detected only after retinoic acid treatment for 2 or more days, suggesting that retinoic acid has no direct inhibitory effect on serum-stimulated c-Fos transcription and that the inhibition is likely the result of changes in the gene expression pattern during endoderm differentiation. In contrast, suppression of c-Fos expression by PD98059 (see below) could be observed in 30 min, and inhibition of F9 cell proliferation could be detected during the first day following the addition of the inhibitor.

**Suppression of c-Fos Expression, but Not MAPK Activation,**

![Figure 3](http://www.jbc.org)
in F9 Cells following Retinoic Acid Treatment—Following serum stimulation of F9 cells, MAPK was activated and remained fairly even for 15–90 min in both retinoic acid-differentiated or undifferentiated cells, as detected in immunoblots using antibodies specific for the phosphorylated/activated kinase (Fig. 3, A and B). On the same blot, a suppression of c-Fos expression by retinoic acid treatment was observed (Fig. 3, A and B). In >10 experiments, we consistently observed a suppression of c-Fos expression in F9 cells upon retinoic acid-induced differentiation, but no inhibition of serum-stimulated MAPK activity. Furthermore, we occasionally observed an enhancement of serum-stimulated MAPK activation in retinoic acid-treated F9 cells, also accompanying reduced c-Fos expression. We observed no inhibitory effect of retinoic acid treatment on Ras and Raf-1 activation by serum stimulation in F9 cells (data not shown), consistent with a previous report (21). Thus, there is a disassociation between MAPK activation and c-Fos expression upon retinoic acid-induced endoderm differentiation of F9 cells.

Dab2, a cell signaling phosphoprotein (54), is a marker for primitive endoderm cells (17, 18) and a substrate of MAPK.\(^2\) Dab2 was present in retinoic acid-treated cells (Fig. 3A), indicating the differentiation of the F9 cells when treated with retinoic acid. Following serum stimulation, progressive retardation of Dab2 migration on the SDS-polyacrylamide gel could be observed. This retardation is due to Dab2 phosphorylation by MAPK (54),\(^2\) consistent with the unsuppressed activation of MAPK in retinoic acid-treated F9 cells.

In both native and differentiated F9 cells, the MAPK isoform ERK2 is much more abundant than ERK1. This distribution differs in cells originating from adult animals such as the MOV mouse ovarian surface epithelial cell line, in which both the ERK1 and ERK2 isoforms of MAPK were found in near equal amounts (Fig. 3D).

Inhibition of Elk-1 Phosphorylation following Retinoic Acid-induced Differentiation of F9 Cells—Activated MAPK phosphorylates the transcription factor Elk-1 at serine 383, and phosphorylated Elk-1 binds with the ternary complex to the c-fos promoter and activates transcription (36). Upon serum stimulation of F9 cells, the phosphorylation of Elk-1 was found to be maximal at 30 min (Fig. 3, A and B). We found that serum-stimulated Elk-1 phosphorylation was much reduced in retinoic acid-induced F9 cells (Fig. 3, A and B), despite a similar MAPK activity in treated and untreated cells. Additionally, the protein level of total Elk-1 was unaltered following retinoic acid-induced differentiation (Fig. 3C). In an immunocomplex of anti-Elk-1 antibodies, the phosphorylation of Elk-1 at serine 383 was much reduced following retinoic acid treatment, despite a similar amount of total Elk-1 protein found in Elk-1 immunoprecipitates from either differentiated or undifferentiated cells (Fig. 3C). Thus, the direct target of retinoic acid treatment in the MAPK pathway and suppression of c-Fos is the reduction of Elk-1 phosphorylation.

Comparison of Suppression by Retinoic Acid-induced Differentiation and Inhibition by PD98059—Upon serum stimulation, a strong activation of MAPK (ERK1/2) was observed; however, no detectable JNK and p38MAPK\(^2\) activities were observed in retinoic acid-induced or undifferentiated control F9 cells (data not shown). The MAPK/MEK inhibitor PD98059 inhibited both Elk-1 phosphorylation (Fig. 4A) and c-Fos expression (Fig. 4B). However, unlike the effect of retinoic acid-induced differentiation, PD98059 also eliminated serum-stimulated MAPK activation (Fig. 4A). Inhibition by PD98059 indicates that MAPK phosphorylation of Elk-1 is essential for serum-stimulated c-Fos expression in F9 cells. As a control, the p38MAPK inhibitor SB202190 did not inhibit MAPK activation, Elk-1 phosphorylation, or c-Fos expression (Fig. 4), indicating that p38MAPK activation is not required for serum-stimulated c-Fos expression in F9 cells. Thus, stimulation of MAPK activity appears to be necessary and sufficient for Elk-1 phosphorylation and c-Fos expression in undifferentiated F9 cells. In differentiated F9 cells, however, MAPK activation is not correlated with Elk-1 phosphorylation and c-Fos expression, indicating that retinoic acid induces an uncoupling of MAPK activity and Elk-1 phosphorylation/activation.

\(^2\) E. R. Smith, W.-P. Sun, J. D. Lambeth, and X.-X. Xu, submitted for publication.

**Fig. 4.** Effect of PD98059 and SB202190 on MAPK activation, Elk-1 phosphorylation, and c-Fos expression in F9 cells. F9 cells were cultured for 4 days with retinoic acid or Me\(_2\)SO solvent control. Cells were then cultured in medium without serum containing 1% bovine serum albumin and 1 \(\mu\)M retinoic acid or Me\(_2\)SO solvent control. The inhibitors PD98059 (25 \(\mu\)M) and SB202190 (25 \(\mu\)M) were added to cells for 30 min prior to stimulation with 15% FBS. A, cell lysates were prepared following 0 or 15 min of stimulation with FBS in SDS gel loading buffer. The lysates were used to determine Elk-1 phosphorylation and MAPK activation by immunoblotting using anti-phospho-Elk-1 (P-Elk-1) and anti-phospho-MAPK (P-Erk) antibodies sequentially. The same blot was then used to determine \(\alpha\)-actin as a loading control. B, cell lysates were prepared following 0 or 90 min of stimulation with FBS, and c-Fos expression was determined by Western blotting. The same blot was then used to determine \(\beta\)-actin as a loading control. Shown is a representative example of three separate experiments, with similar results.
ionomycin (1 μM) used, it appeared that the phospho-Elk-1 level was slightly decreased in Me2SO-treated control cells (Fig. 5B); however, no suppression of c-Fos expression could be detected in the same experiment (Fig. 5A). Thus, the use of ionomycin to activate PP2B does not mimic the effect on c-Fos expression during retinoic acid-induced endoderm differentiation in F9 cells.

Cyclosporin A is a specific inhibitor of PP2B (59, 61). If retinoic acid treatment increases PP2B activity, leading to suppression of Elk-1 phosphorylation and c-Fos expression, cyclosporin A should cause an elevation or restoration of phosphorylated Elk-1 levels and c-Fos expression in differentiated F9 cells. We found that inhibition of PP2B by cyclosporin A did not enhance serum-stimulated c-Fos expression (Fig. 6A), Elk-1 phosphorylation (Fig. 6B), or MAPK activation (Fig. 6, A and B) in either undifferentiated or differentiated F9 cells. In contrast, cyclosporin A appeared to slightly reduce c-Fos expression at high doses. Thus, the response of either undifferentiated or differentiated F9 cells to ionomycin and cyclosporin differs from that of COS-7 and 293 cells, which are responsive regarding c-Fos expression to these agents (56–58, 67). Therefore, we conclude that the uncoupling of MAPK activation and c-Fos expression by retinoic acid-induced F9 differentiation is at the step of inhibition of Elk-1 phosphorylation (Fig. 7). However, decreased Elk-1 phosphorylation is not due to an increase in PP2B activity following retinoic acid-induced differentiation in F9 cells. Thus, through an unclear mechanism without the participation of PP2B activation, MAPK activation and Elk-1 phosphorylation are disassociated in F9 cells following retinoic acid-induced primitive endoderm differentiation, resulting in suppression of c-Fos expression and correlating with cell growth reduction.

**DISCUSSION**

F9 cells are undifferentiated, with characteristics resembling those of stem cells in early embryos, and have been widely used to study early embryonic development and retinoic acid regulation (9–11). Retinoic acid induction of cell differentiation is usually accompanied by reduced cell proliferation, due to suppression of cell cycle progression (62, 63). The growth-suppressive activity of retinoic acid in F9 cells is retinoic acid receptor-β-dependent (10). Although the mechanism for growth suppression by retinoic acid is not yet certain, several possibilities have been investigated. One possibility is that retinoic acid suppresses cell growth by the induction of the transforming growth factor-β pathway (64). Many studies have also indicated that retinoic acid treatment inhibits AP-1 activity (52, 65, 66). Here, we found that retinoic acid treatment reduced serum-stimulated c-Fos expression. Surprisingly, retinoic acid treatment had no effect on MAPK activation, thus uncoupling MAPK activation and c-Fos expression. The effect of retinoic acid has been determined to be on the phosphorylation and activation of Elk-1, a transcription factor required for c-Fos expression (Fig. 7).

A few studies have previously shown uncoupling of MAPK activation and Elk-1 phosphorylation/activation (56, 67, 68). First, transfection of the KSR mammalian ortholog of *Drosophila* KSR can inhibit Elk-1 phosphorylation and activation without affecting MAPK activity (56). The effect of KSR was shown to be through the activation of PP2B (calcineurin), the major phosphatase for Elk-1 (57, 58). The mechanism for the activation of PP2B by KSR has not been defined. A second example is the Grb2-binding adapter protein Gab2, the likely mammalian ortholog of *Drosophila* DOS. Gab2 uncouples MAPK activity...
and Elk-1 phosphorylation/activation when transfected into mammalian cells, although the mechanism is yet unknown (67). A third example is /H9251-synuclein, a chaperon protein that has been identified as a component of Lewy bodies in Parkinson’s disease and diffuse Lewy body disease (68). Expression of /H9251-synuclein prominently attenuates Elk-1 phosphorylation without inhibiting MAPK (68). /H9251-Synuclein is associated with both MAPK and Elk-1 and presumably imposes upon the kinase and substrate a nonproductive conformation (68). Finally, another Grb2-binding protein (53), Dab2, can uncouple MAPK activation and c-Fos expression when transfected into tumor cells (69). Dab2 expression is often lost in tumor cells (70), and re-expression of Dab2 suppresses cell growth and tumorigenicity (55, 71), suggesting a tumor suppressor function. However, the mechanism for the action of Dab2 in uncoupling MAPK activation and c-Fos expression is also unknown. It is possible that Dab2 mediates the retinoic acid-induced uncoupling of MAPK activation and c-Fos expression and the suppression of cell growth since Dab2 is induced by retinoic acid in F9 cells (Fig. 3A) (17). We have attempted transfection to establish Dab2 expression in F9 cells; however, none of the G418-resistant clones were found to have significant Dab2 expression.

Several additional genes including collagen IV are induced along with dab2 by GATA-6 during primitive endoderm differentiation (17–19). Laminin is also induced directly by retinoic acid in F9 cells during retinoic acid-induced differentiation (10, 20). We speculate that expression of dab2 coordinately with several additional genes achieves a balanced signal during F9 cell differentiation; thus, expression of Dab2 alone (without additional working partners) is likely to be incompatible for cell maintenance and growth, which may account for the inability

![Fig. 7. Disassociation of c-Fos expression and MAPK activation in retinoic acid-induced F9 cell differentiation.](http://www.jbc.org/)

| CyA (µM) | DMSO | + RA |
|----------|------|------|
|          | 0    | 0.001| 0.01 | 0.1  | 1    | 10   |
| FBS      |     |      |      |      |      |      |
| FBS -    | +    | +    | +    | +    | +    | +    |
| c-fos    | 65   | 56   | 56   | 56   | 56   | 56   |
| P-Erk    | 40   | 40   | 40   | 40   | 40   | 40   |
| Actin    |      |      |      |      |      |      |

**FIG. 6.** Effects of cyclosporin A on serum-stimulated c-Fos expression and MAPK activation. F9 cells were cultured for 4 days with 1 µM retinoic acid (RA) or MeSO (DMSO) solvent control. The PP2B inhibitor cyclosporin A (CyA) was added at 1–10 µM for 60 min prior to stimulation with 15% FBS. A, cell lysates were prepared 90 min following serum stimulation. A mixture of anti-c-Fos and anti-phospho-ERK (P-Erk) antibodies was used to determine c-Fos expression and MAPK activation by Western blotting. The membrane was blotted with anti-β-actin antibodies as a loading control. B, cell lysates were prepared 30 min following serum stimulation. Elk-1 phosphorylation (P-Elk-1) was determined by Western blotting. Subsequently, MAPK activity was determined by Western blot on the same membrane.

**FIG. 7.** Disassociation of c-Fos expression and MAPK activation in retinoic acid-induced F9 cell differentiation. Shown is a schematic illustration of the effect of retinoic acid (RA), PD98095, ionomycin, and cyclosporin A on MAPK activation, Elk-1 phosphorylation, and c-Fos expression. Retinoic acid-induced F9 cell differentiation results in the inhibition of c-Fos expression due to inhibition of Elk-1 phosphorylation. However, retinoic acid treatment does not inhibit MAPK activation, unlike the action of PD98095. PP2B may dephosphorylate Elk-1 and suppresses c-Fos expression. Cyclosporin A inhibits PP2B and may enhance Elk-1 activity and c-Fos expression, whereas ionomycin activates PP2B and may decrease Elk-1 phosphorylation and suppress c-Fos expression. The lack of inhibition of c-Fos expression by ionomycin in undifferentiated F9 cells and the lack of a stimulating effect on c-Fos expression by cyclosporin A in retinoic acid-treated F9 cells suggest that PP2B is not involved in the regulation of Elk-1 phosphorylation and c-Fos expression in F9 cells during endoderm differentiation.
to isolate Dab2-transfected F9 cells.

There are several possible mechanisms to be considered for how retinoic acid treatment acts to uncouple MAPK and Elk-1. First, retinoic acid may act to dephosphorylate Elk-1, similar to KSR in activating PP2B (56). Retinoic acid may do so by inducing calcium influx or recruiting PP2B to a particular cellular location close to Elk-1. PP2B does not appear to be responsible for the retinoic acid-induced suppression of Elk-1 phosphorylation and activation since the specific inhibitor (cyclosporin A) location close to Elk-1. PP2B does not appear to be responsible for calcium influx or recruiting PP2B to a particular cellular location.

First, retinoic acid may act to dephosphorylate Elk-1, similar to KSR in activating PP2B. Thus, the response of either undifferentiated or differentiated F9 cells to PP2B activity, we have tested ionomycin in a wide range of concentrations since, at low doses, ionomycin may inhibit PP2B, but it also may activate the Ras/MAPK pathway at higher doses (59, 60). Thus, the response of either undifferentiated or differentiated F9 cells to ionomycin and cyclosporin A is different from that of COS-7 and 293 cells, which are responsive regarding c-Fos expression.

uncoupling of MAPK activation and c-Fos expression during endoderm differentiation may enable the cells to attenuate the mitogenic signal of MAPK through Elk-1 and c-Fos, leaving an unsuppressed MAPK activity in endoderm cells. Whether c-Fos expression is critical in F9 cells for mitogenic signaling is not certain. Nevertheless, changes in the ability of serum to activate c-Fos expression accompany F9 cell differentiation into endoderm-like cells and provide a mechanism for the different interpretation and response of the serum signal of the differentiated cells. It is likely that there are many substrates for MAPK beside Elk-1 in primitive endoderm cells. Activated MAPK probably is needed for other processes such as further cell differentiation, migration, cell-cell and cell-matrix interactions, and structural organization. Our finding that retinoic acid-induced F9 cell differentiation into primitive endoderm cells uncouples MAPK activity and Elk-1 phosphorylation (Fig. 7) adds additional complexity and flexibility to the regulation of the Ras/MAPK pathway.

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