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13. Abstract *(Maximum 200 words)* *(abstract should contain no proprietary or confidential information)*

Loss of Disabled-2 (Dab2) expression is frequently an early event in the tumorigenicity of breast and ovarian epithelial cancers. Retinoic acid (RA) treatment of F9 embryonic carcinoma cells induces Dab2 expression in both a time- and concentration-dependent manner, suggesting Dab2 may be integral to the RA-mediated differentiation and development pathway. These studies undertook to investigate whether RA may mediate Dab2 expression in mammary epithelial cells, thereby inducing a negative regulator of the Ras/MAPK pathway. The ability of RA to induce Dab2 in breast carcinoma cell lines was assessed; however, in no case did RA induce expression or alter the growth properties in these cell lines. Presumably an upstream regulator of the RA pathway and/or Dab2 is deficient in these cells. Studies in F9 cells showed that Dab2 uncoupled MAPK activation from its downstream activation of c-Fos, and the affected step was the activation/phosphorylation of the transcription factor, Elk-1. Thus, the loss of Dab2 in tumor cells may contribute to malignancy by removing a negative regulator of MAPK and c-Fos expression.

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INTRODUCTION:

Expression of Disabled-2 (Dab2) is frequently lost in breast and ovarian epithelial cancers. Immunohistochemical staining has shown that this loss is an early event in tumorigenicity, since Dab2 is found absent in premalignant and otherwise morphologically normal tissue adjacent to malignant tissue. Retinoic acid (RA) treatment of F9 embryonic carcinoma cells induces Dab2 expression in both a time- and concentration-dependent manner, suggesting that Dab2 may be integral to retinoid acid signal transduction in differentiation and development. The purpose of the studies undertaken by the proposed research was to test the hypothesis that RA regulates Dab2 expression in breast epithelial cells, thereby inducing a negative growth regulator. Whether Dab2 expression and activation is one mechanism by which retinoids affect proliferation of mammary tumor cells, and the potential of retinoids as chemopreventive agents of mammary cancer, was to be explored. The studies had two basic aims: 1) to determine the effect of retinoic acid on Dab2 expression in RA-sensitive breast cancer cell lines (MCF-7, T47D, SK-Br-3) compared to RA-resistant breast cancer cell lines (MDA-MB-231, MDA-MB-468); and 2) to determine the role of Dab2 in retinoic acid-sensitive growth control.

BODY:

Based on the original hypothesis, we evaluated the ability of retinoic acid to induce Dab2 in the breast carcinoma cell lines, MCF-7, T47D, and SK-Br-3. In no case did retinoic acid induce Dab2 expression in these cells, although retinoic acid did readily induce Dab2 expression in F9 cells, in which four days of treatment with 0.1 μM RA induced Dab2 to levels normally found in the Dab2-expressing immortalized mouse ovarian (MOV) cells. RA also did not alter the growth properties of any of these cells, indicating that an upstream regulator of the RA effect was deficient. The results also suggested that RA by itself may have limited ability to act as a chemopreventive agent in mammary carcinogenesis.

We spent some time trying to develop mammary carcinoma cell lines having inducible Dab2 expression. Mouse and human Dab2 cDNA were separately inserted into the pMT-CB6 vector under the control of the metallothionine promoter. The vectors were individually transfected into MCF-7, as well as F9 cells, using either lipofectamine or electroporation. Clones resistant to G418 were selected and treated with 20-100 μM zinc sulfate, and Dab2 expression was assayed by Western analysis. Despite repeated attempts and transfections, we have been unable to isolate a clone that shows inducible expression of Dab2. This may be due either to the negative growth influence of Dab2 or to some problem with the vector; both of these possibilities are currently being investigated.

Studies using the F9 embryonic carcinoma cell line, however, showed that Dab2 uncoupled MAPK phosphorylation from its downstream activation of c-Fos. The affected step is the activation/phosphorylation of Elk-1, the transcription factor that regulates c-Fos expression. We have some evidence that Dab2 exerts its effect by preventing the translocation of activated MAPK into the nucleus, where it phosphorylates Elk-1. These studies are ongoing.

KEY RESEARCH ACCOMPLISHMENTS:

The research identified the following:

- Retinoic acid does not induce Dab2 expression in mammary carcinoma cell lines, MCF-7, Sk-Br-3, T47D.
- Retinoic acid does not affect the growth of the mammary carcinoma cell lines (above).
- Retinoic acid does induce Dab2 expression in the embryonic carcinoma F9 cells.
- Disabled-2 (Dab2) exerts its tumor suppressive activity by uncoupling c-Fos expression from MAP kinase activation.
- Following retinoic acid-induced differentiation of F9 cells, serum-stimulated MAPK activation and c-Fos expression are uncoupled, similar to the effect seen in Dab2-transfected cells.
- The affected step by Dab2 transfection and RA-differentiation is the phosphorylation/activation of Elk-1.
- Disabled-2 mediates the c-Fos suppression and cell growth regulatory activity of retinoic acid in F9 cells.

REPORTABLE OUTCOMES:

1. He J, Smith ER, and Xu XX. 2001. Disabled-2 exerts its tumor suppressor activity by uncoupling c-Fos expression and MAP kinase activation. *J. Biol. Chem.* **276:** 26814-26818.

2. Smith ER, Smedberg JL, Rula ME, Hamilton TC, and Xu XX. 2001. Disassociation of MAPK activation and c-Fos expression in F9 embryonic carcinoma cells following retinoic acid-induced endoderm differentiation. *J. Biol. Chem.* **276:** 32094-32100.

3. Smith ER, Capo-chichi CD, He J, Smedberg JL, Rula ME, Yang DH, Prowse AH, Godwin AK, Hamilton TC, and Xu XX. 2001. Disabled-2 mediates c-Fos suppression and the cell growth regulatory activity of retinoic acid in embryonic carcinoma cells. *J. Biol. Chem.* **276:** 47303-47310.

4. Smedberg JL, Smith ER, Capo-Chichi DC, Frolov AE, Yang DH, Godwin AK, and Xu XX. 2002. Ras/MAPK pathway confers basement membrane-dependency upon endoderm differentiation of embryonic carcinoma cells. *J. Biol. Chem.* (in press).

5. Smith ER, Smedberg JL, and Xu, XX. 2002. Retinoic acid uncouples MAPK activation and c-Fos expression by regulating nucleocytoplasmic trafficking of Elk-1 in F9 embryonic carcinoma cells. AACR abstract no. 182.

CONCLUSIONS:

Ongoing research indicates that Dab2 may be a regulator of the Ras pathway by uncouping MAPK activation and c-Fos expression. Dab2 is found consistently absent in breast and ovarian carcinoma cells and tissues, and the loss of expression closely correlates with morphological transformation. Our recent data suggests the hypothesis that Dab2 regulates the

activation/phosphorylation of Elk-1 by MAPK. Thus, the loss of Dab2 in tumor cells may contribute to the malignancy by removing a negative regulator of MAPK and c-Fos expression. The biochemical mechanism by which Dab2 may regulate this pathway is under activation investigation, and future experiments will explore the potential interaction of the signaling molecules in endocyting and recycling pathways, as well as in cell-positioning organization.

REFERENCES: None

APPENDICES:

Attached are published articles originating from the research, listed as Reportable Outcomes #1-3.

LIST OF PERSONNEL: Elizabeth R. Smith, Ph.D.

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Disabled-2 Exerts Its Tumor Suppressor Activity by Uncoupling c-Fos Expression and MAP Kinase Activation*

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Disabled-2 (Dab2) is a putative tumor suppressor in breast and ovarian cancers. Its expression is lost in a majority of tumors, and homozygous deletions have been identified in a small percentage of tumors. Dab2 expression is absent or very low in the majority of breast and ovarian cancer cell lines, including MCF-7 and SK-Br-3 breast cancer cells. Transfection and expression of Dab2 in MCF-7 and SK-Br-3 cells suppress tumorigenicity. The cells reach a much lower saturation density and have reduced ability to form colonies on agar plates. In examining the signal transduction pathway of Dab2-transfected cells, we found that serum-stimulated c-Fos expression was greatly suppressed; however, the effects of Dab2 on MAPK family kinases were not as consistent. In MCF-7 and SK-Br-3 cells, although c-Fos expression was suppressed, the Erk1/2, JNK, and p38^{MAPK} activities were unchanged or even increased. Serum-stimulated c-Fos expression is dependent on MAPK/Erk activity because the MEK inhibitor PD98059 suppresses Erk activity and c-Fos expression. Therefore, Dab2 appears to uncouple MAPK activation and c-*fos* transcription. Thus, we conclude that Dab2 re-expression suppresses tumorigenicity by reducing c-Fos expression at a site downstream of the activation of MAPK family kinases. Because Dab2 is frequently lost in cancer, the uncoupling of MAPK activation and c-Fos expression may be a favored target for inactivation in tumorigenicity.

Mitogen-activated kinases (MAPK),[1] also known as extracellular signal-regulated kinases (Erk), are the key downstream targets of the Ras pathway (1–3). The MAPK pathway is used in numerous signaling systems involved in cell growth, differentiation, and development (1–5). The Ras/MAPK pathway has the potential for oncogenic transformation of cells (6, 7), as revealed by the discoveries of viral oncogenes such as v-Ras, v-Raf, v-Jun, and v-Fos. The cellular components of these oncogenes function in the Ras/MAPK pathway. The pathway is also a key target for cell transformation in tumor development in that about 50% of cancers harbor an activating mutation of Ras (6, 8). The cell regulatory system has developed an intricate network for the fine regulation of the Ras/MAPK pathway to counter cell transformation. For example, normal human fibroblasts will undergo senescence or programmed cell death when an activated Ras is introduced (9, 10). Growth factor-stimulated Ras signals are also feedback regulated/inhibited following growth factor binding by receptor degradation (11), dissociation of Sos and Grb2 upon phosphorylation of Sos by activated MAPK (12–15), by the enzymatic actions of Ras GAP, and by the actions of phosphatases specific for the kinases in the pathway. Additionally, the surrounding environment of the cells, such as contact with the extracellular matrix, can modify the effects of growth factors on the activation of Ras/MAPK pathway (16, 17). In breast and ovarian cancers, mutations of Ras are rare, and activating mutations of other components in the Ras/MAPK pathway are also uncommon (18). It is believed that regulators in the fine-tuning of the Ras/MAPK pathway are lost, resulting in aberrant activation of the pathway.

The conservation of the Ras/MAPK pathway in yeast, *Caenorhabditis elegans* and *Drosophila* has helped to delineate the components and regulation of the pathway. In mammalian cells, the growth factor binds to its tyrosine kinase receptor and stimulates its autophosphorylation on its tyrosine residues. The phosphotyrosine residues on the receptor act as a docking site for assembling critical intracellular signaling molecules at the cell membrane to initiate a signal cascade (19, 20). The adapter molecule Grb2 binds to the tyrosine receptor through Shc or directly to the phosphotyrosine residue, bringing associated Sos to activate Ras on the plasma membranes. Ras is activated upon conversion to the GTP bound form and initiates the Raf-1/MEK/MAPK kinase cascade. An established target for MAPK is Elk-1, a transcription factor required for transactivation of c-Fos (21–23). c-Fos was first identified as a cellular counterpart of the viral oncogene capable of cell transformation (24), and its expression is the target of regulation in cell growth control (25). c-*fos* is an immediate early gene whose transcription is activated by serum and growth factors, and its expression is a key switch in cellular regulation (24, 25). c-Fos, together with c-Jun, form the AP-1 transcriptional complex required for the transcription of many genes important for cell growth, differentiation, and transformation (26, 27).

We now report that the expression of c-Fos is a target for the regulatory function of Disabled-2 (Dab2), a candidate tumor suppressor of breast and ovarian tumors (28–30). Dab2, a mammalian ortholog of the *Drosophila* Abl kinase-interacting protein Disabled (31), was isolated as a mitogen responsive phosphoprotein (32). Dab2 was identified by differential displaying to be a gene whose expression was absent in ovarian

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; Erk, extracellular-signal regulated kinase; GST, glutathione S-transferase; Dab2, Disabled-2; JNK, Jun N-terminal kinase; MEK, MAPK or Erk kinase; Sos, Son-of-sevenless; DOS, daughter of sevenless; KSR, kinase suppressor of Ras; GAP, GTP-activating protein; Grb2, growth factor receptor binding 2; PID, phosphotyrosine-interacting domain; PTB, phosphotyrosine binding domain; FBS, fetal bovine serum; Abl kinase, Abelson kinase; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

cancer cells but present in normal ovarian epithelial cells (28). We have previously found that Dab2 is expressed in breast and ovarian epithelial cells, but its expression is lost in the majority (about 85%) of breast and ovarian tumor cells (28, 29, 30). Dab2 contains a phosphotyrosine-interacting domain (PID, or PTB) in its N terminus and a proline-rich, SH3-binding domain in its C terminus, resembling an adapter molecule (32). Its binding to Grb2, competing with Sos, leads to the hypothesis that Dab2 is a Ras/MAPK pathway regulator that is lost in cancer (33). We found that re-expression of Dab2 in breast cancer cells leads to suppression of c-Fos expression and cell growth inhibition. Surprisingly, Dab2 does not inhibit MAPK activity. Thus, a regulatory step in the Ras/MAPK pathway is the uncoupling of the activation of MAPK and transcriptional activation of c-Fos, mediated by Dab2. Tumor cells likely abrogate the essential regulation of the Ras/MAPK pathway in normal cells by the elimination of Dab2, which might contribute to cell transformation.

EXPERIMENTAL PROCEDURES

Materials—Kinase inhibitors, PD98059 and SB202190, were purchased from Calbiochem (San Diego, CA). Tissue culture plastic wares were obtained from Fisher Scientific Inc. (Springfield, NJ). DMEM medium was purchased from Mediatech (Herndon, VA); fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA); antibiotic-antimycotic (100×) solution, LipofectAMINE, and serum-free Opti-MEM I medium were purchased from Life Technologies, Inc. (Grand Island, NY). The ECL Western blot detection kit was purchased from PIERCE (Rockfort, IL); Hybrisol I hybridization solution was from Intergen Inc. (Purchase, NY); positively charged nylon membranes were from Roche Molecular Biochemicals; general chemicals and solvents including Me_2SO, ethanol, isopropyl alcohol, and agarose were from Sigma or Fisher and were of reagent grade or higher.

Cell Culture—MCF-7 and SK-Br-3 human breast cancer cells were purchased from ATCC. The cells were cultured in DMEM with 10% FBS supplemented with 1% non-essential amino acid mix and antibiotic-antimycotic solution.

Antibodies and Western Blot—Anti-Dab2 antibodies were characterized previously (30–32). Anti-p96 antibodies were purchased from Transduction Labs. (Lexington, KY); anti-c-Fos was from UpState Biotechnology (Lake Placid, NY); anti-β-actin was from Sigma; anti-Erk1/2 and phospho-Erk1/2 were from Biolab and Cell Signaling Technology Inc. (Beverly, MA); anti-Elk-1 and anti-phospho-Elk-1 were from Promega and Santa Cruz Biotechnology (Santa Cruz, CA). Western blotting was performed according to standard procedures, as described previously (33). In some cases, after gaining experience with usage of a single antibody, two or more antibodies were used in the same incubation to detect various molecular weight proteins simultaneously.

Cell Transfection—The full-length human DAB2 cDNA (GenBank™ accession number AF188298) was inserted into the pcDNA/zeo eukaryotic expression vector (Invitrogen, La Jolla, CA). Plasmid DNA was purified using the Qiagen Maxiprep column, and LipofectAMINE reagent was used for transfection. Briefly, 2 μg of Dab2 expression construct or vector control plasmid DNA was mixed with 20 μl of LipofectAMINE in 1 ml of Opti-MEM and was added to cells for 16 h. The transfection mix was removed, and fresh DMEM containing 10% FBS was added. After 12 h, selection medium (DMEM with 10% FBS and 300 ng/ml of Zeomycin) was added to the cells. Following a 10–12 day selection with change of medium every 2 days to removed dead cells, selected clones were isolated and collected by cloning rings, expanded by further culturing, and examined for Dab2 expression by Western blotting.

Cell Growth Analysis—Cell growth was determined by counting under a microscope and the MTT assay (Promega). For cell counting, cells (around 10^5 per plate) were plated in 35-mm tissue culture dish (6-well dish), and medium was changed daily. At the indicated times, cells were harvested in 1 ml of trypsin-EDTA, collected by centrifugation and resuspended in 100 μl of phosphate-buffered saline for counting. For the MTT assay, cells (around 10^4) were plated in 96-well plates, and medium was changed daily. On the indicated day, the MTT dye was added and incubated with the cells for 2 h in the tissue culture incubator. The reaction was stopped by addition of cell solubilization solution. After 2 h at room temperature, the absorbance of the solution at 570 nm was measured with a microplate reader.

Colony Formation on Agar Plates—Cells were embedded in a 0.3% low melting point agarose top layer in culture medium, plated on top of a 0.6% agarose bed in DMEM containing 10% FBS and complete supplements. The agarose plates were incubated at 37 °C for 3 weeks, with addition of fresh medium every 3 days.

Cell Cycle Analysis by Flow Cytometry—Cells on 100-mm plates were harvested with trypsin-EDTA solution and pelleted by centrifugation. The cells were then fixed with 70% ethanol, pelleted, and resuspended in propidium iodine staining solution for 30 min at 4 °C. The stained cells were analyzed by flow cytometry.

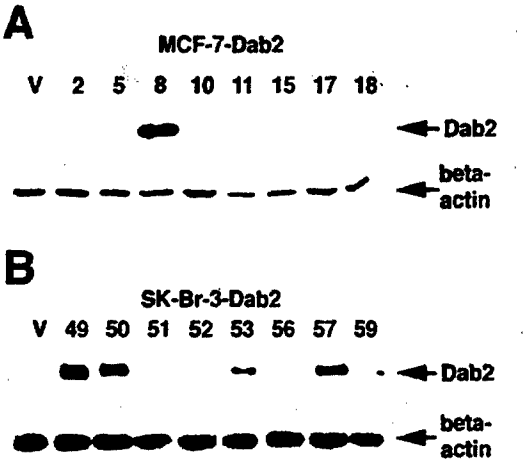

FIG. 1. **Establishment of Dab2-transfected MCF-7 and Sk-Br-3 breast cancer cells.** MCF-7 (*A*) and Sk-Br-3 (*B*) breast cancer cells on 35-mm wells were transfected with human Dab2 cDNA expression construct or pcDNA3 vector alone using LipofectAMINE. Two days following transfection, the cells were transferred to 100-mm plates and cultured in medium with 300 ng/ml Zeomycin. The medium was changed every 2 days to remove floating dead cells, and Zeomycin-resistant colonies formed were harvested using cloning cylinders and plated on 24-well plates. The cell cultures were expanded, and a fraction was used for analysis for Dab2 expression by Western blot. β-actin was determined on the same blot as a protein-loading control.

RESULTS

Establishment of Dab2 Expression in MCF-7 and SK-Br-3 Breast Cancer Cells—We have previously found that Dab2 is expressed in breast and ovarian epithelial cells, but its expression is lost in the majority (about 85%) of breast and ovarian tumor cells tested (28, 29, 30). Forced expression of Dab2 in tumor cells reduces cell growth, induces cell death, and suppresses tumorigenicity in the nude mouse xenograft model (29, 34). To further determine the biological consequence of Dab2 loss for the tumor cells and to examine the signal transduction pathway affected, we have transfected and established Dab2 expression in MCF-7 and Sk-Br-3 breast tumor cells. Dab2 expression is absent in these two breast carcinoma cell lines (30).

Following transfection of the human Dab2 cDNA in the pcDNA expression vector into MCF-7 breast cancer cells, 26 zeomycin-resistant clones were selected. Of these clones, only two clones were found to express Dab2 as detected by Western blot, and a single clone (clone 8) still retained Dab2 expression upon expansion of the cells in culture (Fig. 1*A*). In contrast, 22 of 64 Sk-Br-3 clones selected retained some expression of the transfected Dab2, and the three high expressing clones 49, 50, and 57 were chosen for further analysis (Fig. 1*B*). Three randomly selected vector transfected clones of each cell line were expanded for use as controls.

Transfection and Expression of Dab2 Inhibit Cell Growth and Transformation in MCF-7 and Sk-Br-3 Breast Cancer Cells—Upon establishment of the Dab2-expressing cells, we first characterized the growth properties of the cells. Transfected MCF-7 cells (clone 8) were found to grow more slowly in either low (0.1%) or high (10%) serum compared with vector-transfected controls (Fig. 2*A*). A similar growth retardation

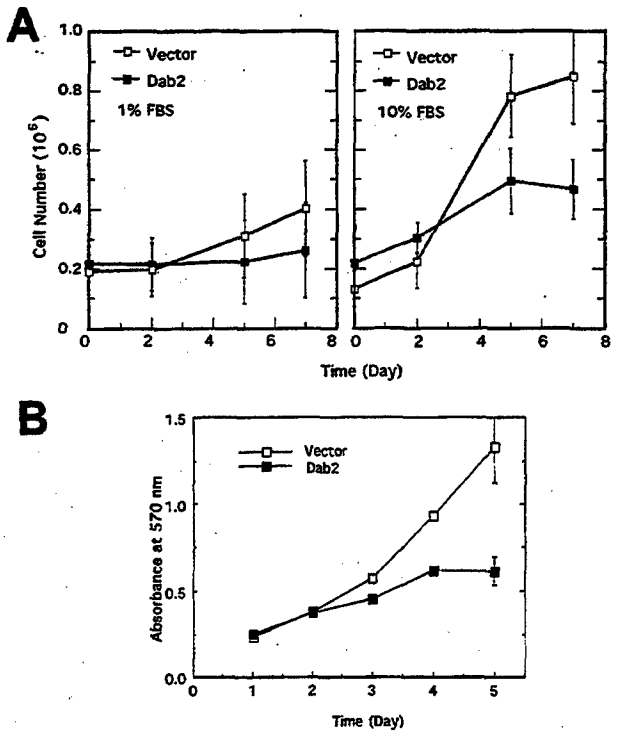

FIG. 2. **Characterization of MCF-7 and Sk-Br-3 cell clones transfected with Dab2.** Growth curve for MCF-7 vector-transfected or Dab2-transfected clone 8 (*A*) and SK-Br-3 vector-transfected or Dab2-transfected clone 49 (*B*) cells. Cells on 35-mm plates were cultured in medium with 1 or 10% serum. Cell numbers were determined by counting (*A*) or MTT assay (*B*). *Error bars* indicate S.D. from measurement of triplicate plates. Data shown are representative of five or more independent experiments using either cell counting or the MTT assay.

TABLE I

Cell cycle parameters of the transfected cell clones

MCF-7 and Sk-Br-3 non-transfected, pcDNA3 vector-transfected, and Dab2-expressing clones were grown to 80% confluency on 10-mm plates in medium containing 10% fetal bovine serum. Cells were then detached by trypsin digestion and subjected to flow cytometry analysis using propidium iodine staining to determine percentage of cells in various stages of the cell cycle. Duplicate plates of cells for each clone were used in each experiments, and the variation between the result of the duplicates was smaller than 10%. Similar results were obtained from three independent experiments, and data from a representative experiment are shown.

Cell clone	G_0/G_1	S	G_2/M
	Cell cycle distribution		
	%		
MCF-7	66	16	18
MCF-7-Vector	64	15	21
MCF-7-Dab2 (no. 8)	76	9	15
SK-Br-3	44	8	48
SK-Br-3-vector	52	10	38
SK-Br-3-Dab2 (no. 49)	71	3	26
SK-Br-3-Dab2 (no. 50)	63	3	34
SK-Br-3-Dab2 (no. 57)	69	4	27

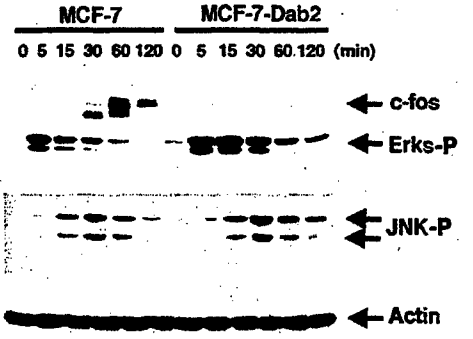

FIG. 3. **Effect of Dab2 expression on activation of Erk1/2, JNK, and $p38^{MAPK}$ kinases following serum stimulation.** MCF-7 cells transfected with vector or Dab2 (clone 8) were seeded on 35-mm plates. The cells were cultured without serum for 18 h and then stimulated with serum for 0, 15, 30, 60, and 120 min. Cells were immediately washed twice with cold phosphate-buffered saline, lysed with SDS gel loading buffer and boiled for 5 min. The cell lysates were analyzed by Western blotting for MAPK activation with anti-phosphopeptide antibodies for Erk1/2, JNK, and $p38^{MAPK}$. Expression of c-Fos was determined simultaneously on the same blot as MAPK activation. β-Actin was used as a loading control.

was found in the three Dab2-expressing SK-Br-3 clones compared to three vector-transfected clones (Fig. 2*B*). The Dab2-transfected MCF-7 cells were found to have a reduced ability to form colonies on agar plates; MCF-7-Dab2 clone 8 cells formed fewer (about 20% of control) and smaller colonies than vector-transfected clones (data not shown). All three clones of Dab2-transfected Sk-Br-3 cells also had a reduced ability to form colonies on agar plates (not shown). All four selected Dab2-expressing tumor cell clones were analyzed for cell cycle parameters using flow cytometry (Table I). Compared with vector-transfected and non-transfected cells, both MCF-7 and Sk-Br-3 cells expressing Dab2 have about a 50% lower percentage of cells in S phase and about 25% lower percentage of cells in G_2/M phase, suggesting a prolonged G_1 phase. These results are consistent with previous reports with different tumor cell lines on the negative cell growth regulatory properties and tumor suppressive activity of Dab2 (29, 35). Our goal is to analyze the effects on the cellular signal transduction pathways by the Dab2 protein in these cells.

Dab2 Transfection and Expression Inhibits Serum-stimulated c-Fos Expression—Next, we examined the possible changes in mitogenic signaling in Dab2-expressing cells compared with vector-transfected cells. We observed by both Western and Northern blots a reduction of c-Fos expression upon serum stimulation of the Dab2-expressing cells. For both MCF-7 cells and Sk-Br-3, c-Fos is induced by serum at 30 min and is maximal at 60 min in vector-transfected cells (and also in non-transfected cells). In Dab2-expressing MCF-7 cells (clone 8) (Fig. 3) and a representative clone 49 of Dab2-expressing SK-Br-3 cells (Fig. 5*B*), little c-Fos expression is induced by serum. The same effect of Dab2 expression on c-Fos expression was also observed in transfection of tumor cells with Dab2 using an adenoviral vector in our previous investigation (34). Thus, the suppression of c-Fos expression is not because of the particular properties of the selected cell clones but the result of Dab2 expression.

Effect of Dab2 Expression on the Activation of Erk1/2, JNK, and $p38^{MAPK}$ Kinases following Serum Stimulation—It has been established that MAPK activation leads to c-Fos transcription and increases in AP-1 activity (21–23). To our surprise, however, no reduction in MAPK activity was observed in Dab2-expressing MCF-7 or SK-Br-3 cells although c-Fos expression was suppressed. To eliminate possible artifacts and to confirm this observation, we performed Western blot analysis to determine c-Fos expression and MAPK activation simultaneously on the same blot by including a proper mix of anti-c-Fos and anti-phospho-MAPK (activated) antibodies in the same incubation. c-Fos expression and MAPK activation by serum were determined for clone 8 Dab2-expressing MCF-7 cells (Fig. 3), and a representative clone 49 of Dab2-expressing Sk-Br-3 cells (Fig. 5*B*). In the Dab2 expressing MCF-7 cells, though

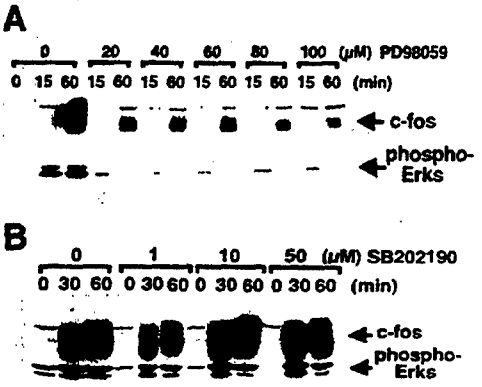

FIG. 4. **Inhibition of serum-stimulated c-Fos expression by PD98059 and SB202190 in MCF-7 cells.** MCF-7 cells were seeded onto 35-mm plates and grown to 80% confluency and were then cultured without serum for 18 h. By the end of the 18-h incubation, serial concentrations of PD98059 (*A*) or SB202190 (*B*) were added and incubated for 30 min. The cells were then stimulated with 10% serum in the presence of the same concentration of the compounds. Cell lysates were prepared at 0, 15 (or 30), and 60 min time points for Western blot analysis using antibodies for c-Fos and phospho-Erk1/2 to determine c-Fos expression and MAPK activation simultaneously.

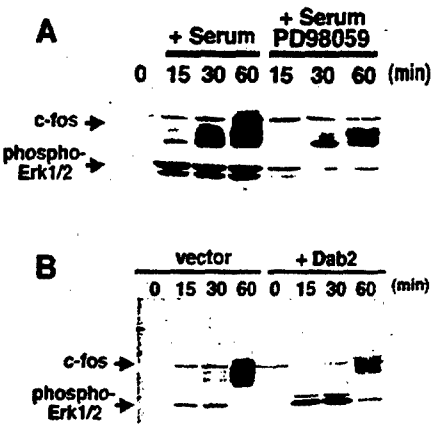

FIG. 5. **Comparison of Dab2 expression and PD98059 on the inhibition of serum-stimulated c-Fos expression in SK-Br-3 cells.** *A*, SK-Br-3 cells (untransfected) were seeded onto 35-mm plates and were cultured without serum for 18 h. By the end of the 18-h incubation, PD98059 (100 μM) was added to one set of cells for 30 min. The cells were then stimulated with 10% serum, with or without addition of PD98059 (100 μM) for 0, 15, 30, and 60 min. The cell lysates were analyzed for c-Fos expression and MAPK activation by Western blotting. *B*, SK-Br-3 cells transfected with vector or Dab2 (clone 49) were seeded onto 35-mm plates, and were cultured without serum for 18 h. The cells were then stimulated with 10% serum. Cell lysates were harvested at 0, 15, 30, and 60 min and were assayed for c-Fos expression and MAPK activation simultaneously by Western blot analysis.

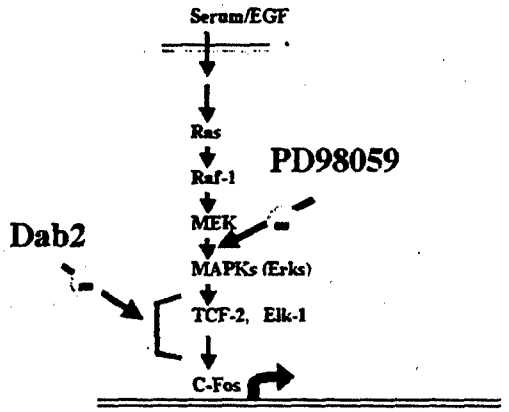

FIG. 6. **Schematic model presentation of Dab2 regulation of c-Fos expression.** Serum and growth factor activates Ras and MAP kinase, which is inhibited upon addition of the MEK inhibitor PD98059. Phosphorylation of Elk-1 by MAPK is required for serum-stimulated c-Fos transcription. The pathway is regulated by Dab2 by uncoupling MAPK activation and c-Fos expression. This regulatory mechanism is often absent in tumor cells because of the loss of Dab2.

serum-stimulated c-Fos expression is greatly reduced compared with vector-transfected control, MAPK activity is enhanced as detected by phosphopeptide antibodies in this experiment (Fig. 3). Similar effects of Dab2 expression in SK-Br-3 on c-Fos expression and MAPK activation were found in Dab2-expressing clones (not shown). We also investigated and found no effect of Dab2 expression on Ras and Raf-1 activation (not shown). Thus, restoration of Dab2 expression appears to dissociate MAPK activation and c-Fos expression in tumor cells.

The effect of Dab2 expression on other MAPK family kinases including JNK and $p38^{MAPK}$ kinases was also investigated. Dab2 expression appears to have no significant and consistent effect on serum-stimulated JNK activity as detected by Western blot with JNK-phosphopeptide specific antibodies (Fig. 3). In either Dab2-expressing or vector-transfected MCF-7 and SK-Br-3 cells, serum stimulation did not notably activate $p38^{MAPK}$ as detected by anti-$p38^{MAPK}$ phosphopeptide antibodies (not shown), though in the same experiment, strong activation was observed in a positive control using anisomycin as a stimulating agent.

Effect of Kinase Inhibitors on c-Fos Expression—The effect of MAPK family kinase activation on c-Fos expression was further explored in MCF-7 and Sk-Br-3 cells using kinase inhibitors PD98059 and SB202190. In MCF-7 cells, the MEK inhibitor PD98059 inhibited MAPK activation and c-Fos expression in a dose-dependent manner (Fig. 4*A*), indicating PD98059-inhibitable MAPK activity is necessary for serum to activate c-Fos expression. In contrast, the $p38^{MAPK}$ inhibitor SB202190, although it appears to reduce the basal state of MAPK activity, had no inhibitory effect on serum-stimulated MAPK (Erk) activation and c-Fos expression (Fig. 4*B*). In comparison, inhibition of c-Fos expression by MEK inhibitor PD98059 (Fig. 5*A*) mechanistically differed from inhibition of c-Fos expression by Dab2 expression (Fig. 5*B*) in SK-Br-3 cells. Thus, Erk1/2, but not $p38^{MAPK}$, is required for serum-stimulated c-Fos expression. Unlike PD98059, Dab2 restrains c-Fos expression at a step between MAPK activation and c-Fos expression without inhibiting MAPK activity (Fig. 6).

Effect of Dab2 Expression on the Phosphorylation/Activation of Elk-1—It has been established that MAPK phosphorylates/activates the transcription factor Elk-1, and activated Elk-1 binds to the c-Fos promoter and activates expression of c-Fos (21–23). We found that Dab2 expression reduced the serum-stimulated phosphorylation of Elk-1 in MCF-7 cells and in Sk-Br-3 cells (not shown). To explore the mechanism for the effects of Dab2 on MAPK and Elk-1, we examined the physical interaction between Erk1/2, Elk-1, and Dab2. In co-immunoprecipitation experiments, we found that Dab2 is not associated in any significant way with Erk1/2 or Elk-1, either the phosphorylated or unphosphorylated proteins. Thus, through an indirect but unclear mechanism, Dab2 uncouples MAPK activation and Elk-1 phosphorylation.

Conclusion—Dab2 is frequently lost in breast and ovarian tumors (30). We have shown here in MCF-7 and SK-Br-3 breast cancer cells, and others have shown in additional tumor cells (29, 35), that transfection and expression of Dab2 suppresses tumorigenicity: the cells reach a much lower saturation density, have reduced ability to form colonies on agar plates, and have suppressed ability to develop tumors in nude mice. In analysis of signal transduction pathways affected, we have found that serum-stimulated c-Fos expression is greatly sup-

pressed. Expression of c-Fos is activated through the action of MAP kinase phosphorylation (21–23). Surprisingly, the Erk1/2, JNK kinase, and $p38^{MAPK}$ activities were unchanged or even increased upon serum stimulation in transfected Dab2-expressing cells compared with vector-transfected cells. Thus, we conclude that Dab2 re-expression suppresses tumorigenicity by uncoupling MAPK activation and c-Fos expression. Although Dab2 could have additional effects on the cells, the suppression of c-Fos expression may be sufficient to suppress cell growth and transformation.

It is well established that the Ras pathway through a cascade of kinases, results in activating the expression of immediate early genes such as c-Fos (21–23). Normally, Ras/MAPK activity is well correlated with c-Fos expression. MAP kinases, Erk1 and Erk2, upon activation will phosphorylate Elk-1, an ETS family transcription factor (21). Phosphorylation of Elk-1 at serine 383 activates its ability to participate in the transcription complex that transcribes c-Fos (21–23). Two recent studies report that MAPK activation and Elk-1 phosphorylation/activation are uncoupled (36, 39). KSR, a mammalian ortholog of the *Drosophila* kinase suppressor of Ras (KSR), can inhibit Elk-1 phosphorylation without affecting MAPK activation (36). The affect of KSR on inhibition of Elk-1 phosphorylation is believed to act through the activation of the Ca^{2+} and calmodulin-regulated PP2B (calcineurin), the major phosphatase for Elk-1 (37, 38). The mechanism for the activation of PP2B by KSR is still unknown. Another report shows that adapter protein Gab2, the probable ortholog of the *Drosophila* daughter of sevenless (DOS), acts to uncouple signaling from MAPK to Elk-1, though no mechanism is yet known (39). It is interesting that both Dab2 and Gab2 are Grb2-binding proteins (32, 39), which may provide some common mechanism in uncoupling MAPK and Elk-1. In normal cells that are Dab2-positive, the ability to uncouple MAPK activation and c-Fos expression will enable the cells to achieve precise control of biological processes, because the Ras/MAPK pathway is widely used for cell growth, differentiation, and development (1–5).

There are several possible mechanisms for Dab2 to uncouple MAPK and Elk-1. First, Dab2 may act to dephosphorylate Elk-1, similar to KSR in activating PP2B. Dab2 may do so by inducing calcium influx or recruiting PP2B to a particular cellular location. Alternatively, Dab2 may inhibit phosphorylation of Elk-1 by MAPK. We have found no physical association between Dab2 and Erk1/2 or Elk-1. Dab2 could still prevent the phosphorylation of Elk-1 by blocking nuclear entry of the activated MAPK or sequestration of Elk-1 from being phosphorylated by the activated MAPK. We are currently investigating these possibilities.

In summary, we have uncovered a regulatory site in the Ras pathway by the candidate tumor suppressor Dab2: the uncoupling of MAPK activation and c-Fos expression (Fig. 6). Suppression of c-Fos expression is consistent with the finding that Dab2 expression retards the progression of the cells through G_1 phase (Table I). Dab2 is lost in the majority of breast and ovarian cancer cells and tissues. Thus, in non-tumorigenic normal cells, the Ras pathway is regulated by Dab2-mediated uncoupling of MAPK activation and c-Fos expression. This appears to be a favored target for inactivation during tumorigenicity; tumor cells eliminate Dab2 and thus a regulatory site in the Ras pathway. The loss of Dab2-mediated regulation of c-Fos expression likely contributes to malignancy.

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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 [1] (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; MTT, 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, Grb2-associated binder-2; DOS, Daughter of Sevenless.

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 Me_2SO, ethanol, isopropyl 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% CO_2 in a humidified tissue culture incubator.

All-*trans*-retinoic acid was dissolved in Me_2SO 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 Me_2SO to make 50 and 6 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 (~10^3 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 were 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.

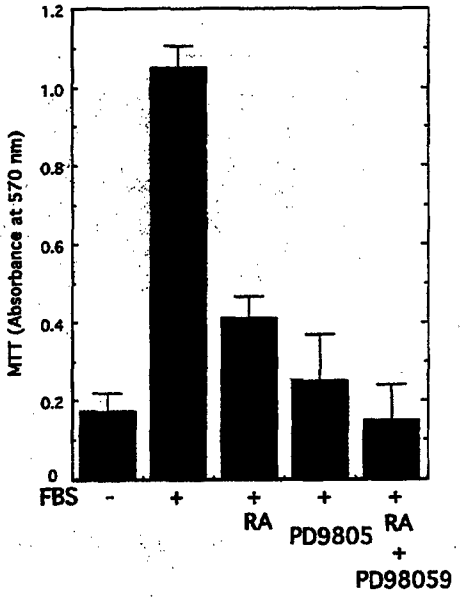

FIG. 1. **Effect of serum, retinoic acid, and the MEK inhibitor PD98059 on the growth of F9 cells.** F9 cells in monolayer were cultured in Dulbecco's modified Eagle's medium for 4 days with or without 10% serum, with 1 μM retinoic acid (*RA*) or Me_2SO solvent control, with or without 25 μM PD98059, or with 25 μM PD98059 plus 1 μM retinoic acid. On the final day, cell numbers were estimated using MTT assay. The absorbance at 570 nm is reported, representing relative cell numbers. Results are reported as the means ± S.D. of triplicate samples. Shown is a representative example of four independent experiments.

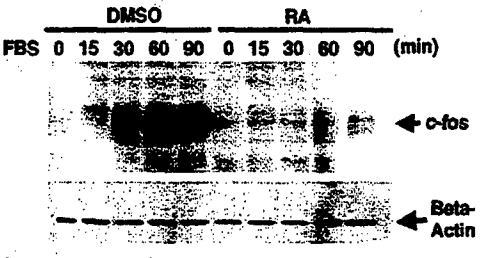

FIG. 2. **Suppression of c-Fos expression in F9 cells following retinoic acid-induced primitive endoderm differentiation.** F9 cells were cultured for 4 days in Dulbecco's modified Eagle's medium containing 10% FBS and 1 μM retinoic acid (*RA*) or Me_2SO (*DMSO*) solvent control. The cells were cultured on the last day without serum and then stimulated with 15% FBS, and cell lysates were used to determine c-Fos and β-actin levels (as a loading control) by Western blotting. These results are representative of >10 independent experiments.

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

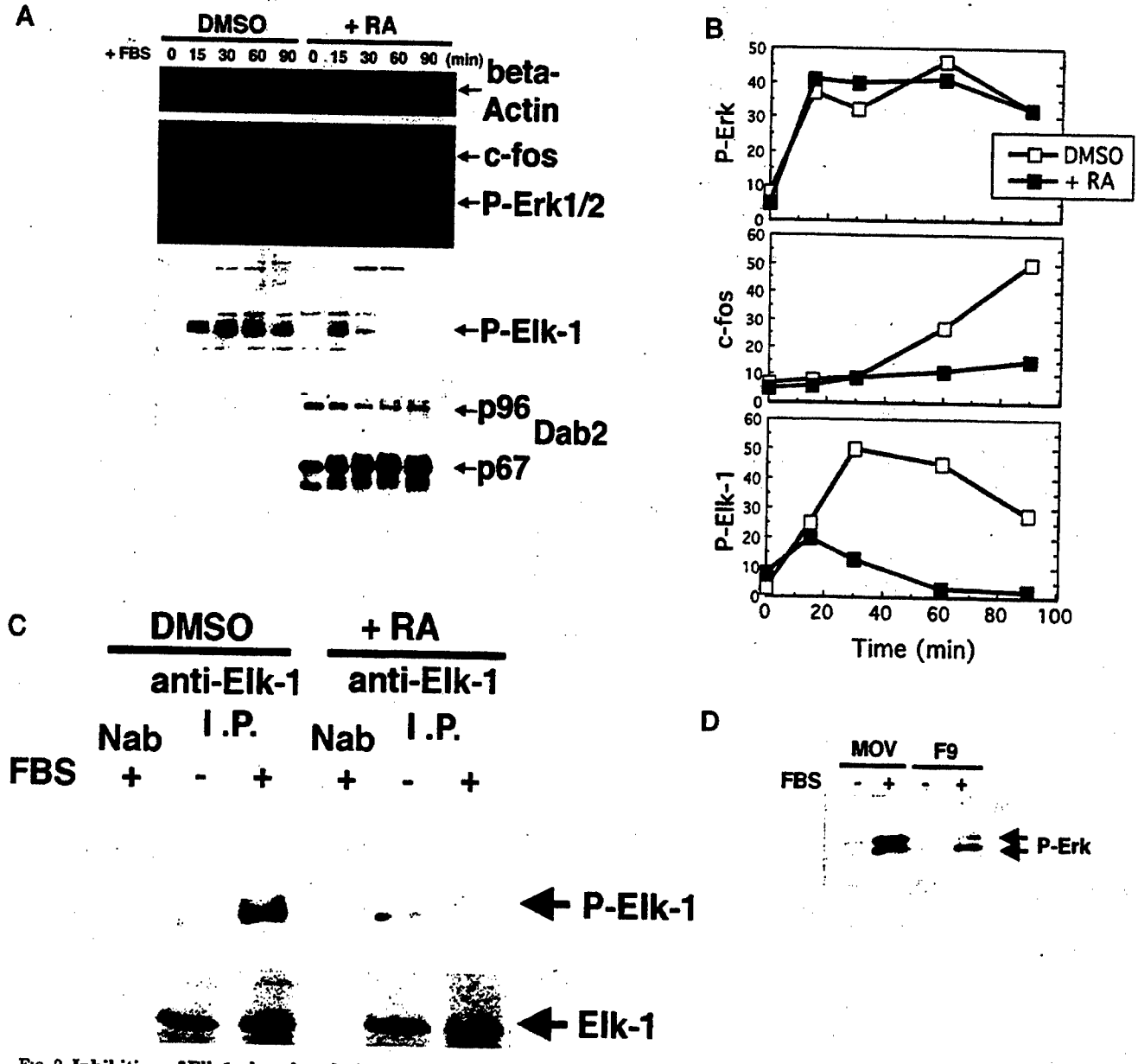

FIG. 3. **Inhibition of Elk-1 phosphorylation, but not cellular MAPK activation, by retinoic acid treatment in F9 cells.** F9 cells were cultured with or without retinoic acid (*RA*; 1 μM) for 4 days and were then stimulated with 15% serum for 0–90 min. *A*, the lysates were analyzed sequentially by Western blotting to determine MAPK activation using anti-phospho-ERK antibodies (*P-Erk1/2*) and to determine Elk-1 phosphorylation using anti-phospho-Elk-1 antibodies (*P-Elk-1*). The same blot was then used to determine Dab2 expression and β-actin protein levels. Anti-c-Fos antiserum was included with anti-phospho-ERK1/2 antibodies to determine c-Fos expression and MAPK activation on the same blot. Similar results were observed in five separate experiments. *B*, the signals from the blot were quantified by densitometry to indicate the relative intensity. *C*, F9 cells treated with Me_2SO (*DMSO*) or retinoic acid were stimulated with serum for 30 min. The cell lysates were used for immunoprecipitation (*I.P.*) with polyclonal anti-Elk-1 antibodies and non-relevant rabbit antiserum (*Nab*) as a control. The immunocomplexes were analyzed by Western blotting using monoclonal anti-phospho-Elk-1 or polyclonal anti-Elk-1 antibodies. *D*, MOV mouse ovarian surface epithelial cells and F9 cells were stimulated with 15% FBS for 15 min. Cell lysates were subjected to Western blotting with anti-phospho-ERK antibodies that recognize both ERK1 and ERK2 equally.

cells cultured in 10% serum. The number of cells cultured in 1 μM retinoic acid or in the presence of PD98059 was 38 or 26%, respectively, of that of control cells cultured in 10% serum.

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,

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. 3*A*), 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. 3*D*).

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. 3*C*). 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. 3*C*). 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 PD98095—Upon serum stimulation, a strong activation of MAPK (ERK1/2) was observed; however, no detectable JNK and $p38^{MAPK}$ 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. 4*A*) and c-Fos expression (Fig. 4*B*). However, unlike the effect of retinoic acid-induced differentiation, PD98095 also eliminated serum-stimulated MAPK activation (Fig. 4*A*). Inhibition by PD98095 indicates that MAPK phosphorylation of Elk-1 is essential for

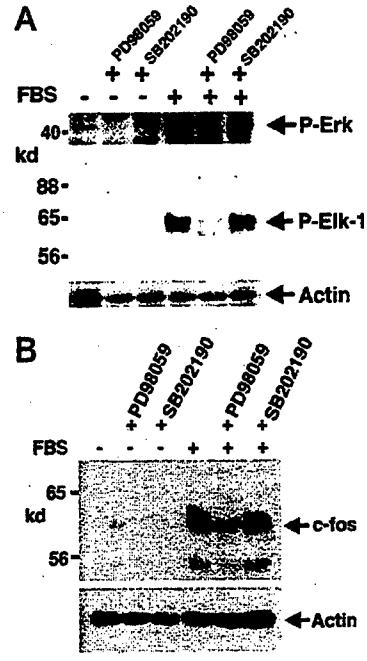

FIG. 4. **Effect of PD98059 and SB202190 on MAPK activation, Elk-1 phosphorylation, and c-Fos expression in F9 cells.** F9 cells were cultured for 4 days with retinoic acid or Me_2SO solvent control. Cells were then cultured in medium without serum containing 1% bovine serum albumin and 1 μM retinoic acid or Me_2SO solvent control. The inhibitors PD98059 (25 μM) and SB202190 (25 μM) 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 α-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 β-actin as a loading control. Shown is a representative example of three separate experiments, with similar results.

serum-stimulated c-Fos expression in F9 cells. As a control, the $p38^{MAPK}$ inhibitor SB202190 did not inhibit MAPK activation, Elk-1 phosphorylation, or c-Fos expression (Fig. 4), indicating that $p38^{MAPK}$ 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.

Retinoic Acid-induced Inhibition of c-Fos Expression Is Not Due to an Increase in PP2B Activity—One precedent for uncoupling of MAPK activation and Elk-1 transcriptional activity is the enhancement of PP2B (calcineurin) activity by KSR (56). PP2B is the major phosphatase for the dephosphorylation of phosphorylated/activated Elk-1, leading to down-regulation of its activity (57, 58). To determine if PP2B is responsible for the reduced phosphorylation of Elk-1 and suppressed expression of c-Fos in retinoic acid-treated F9 cells, we examined the effect of ionomycin (activator of PP2B) in these cells. Cellular second messenger calcium acting through calmodulin (59, 60) can activate PP2B. In laboratory research, the calcium ionophore ionomycin is commonly used to increase cytosolic calcium levels, thus to activate PP2B. The addition of ionomycin had no significant effect on c-Fos expression (Fig. 5*A*), Elk-1 phosphorylation (Fig. 5*B*), or MAPK activation (Fig. 5*C*) in both retinoic acid-treated and untreated F9 cells. At the highest dose of

[2] E. R. Smith, W.-P. Sun, J. D. Lambeth, and X.-X. Xu, submitted for publication.

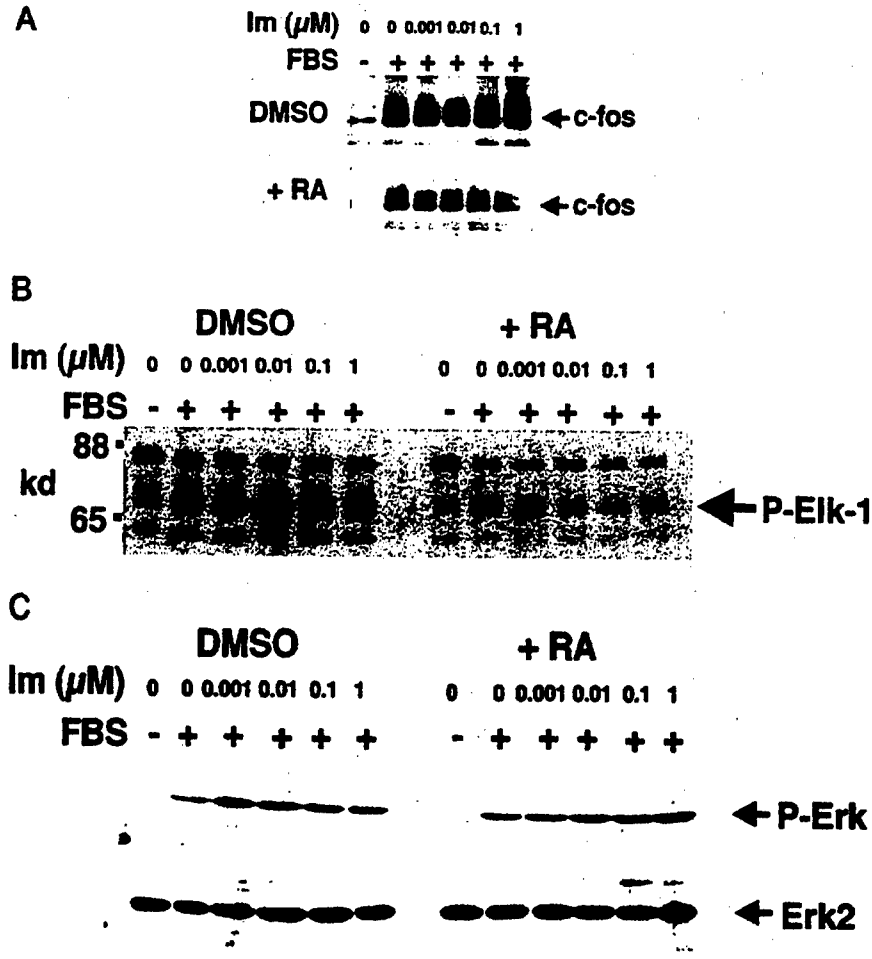

FIG. 5. **Effect of ionomycin on serum-stimulated c-Fos expression, Elk-1 phosphorylation, and MAPK activation in F9 cells.** F9 cells were cultured for 4 days with retinoic acid (*RA*) or Me_2SO (*DMSO*) solvent control. Cells were pretreated for 1 h with 0–1 μM ionomycin (*Im*) and subsequently stimulated with 15% FBS in the continued presence of ionomycin. *A*, cell lysates were harvested 0 or 90 min following serum stimulation and analyzed for c-Fos expression by Western blotting. *B*, cell lysates were harvested 30 min following FBS stimulation and used to determine Elk-1 phosphorylation by Western blotting using anti-phospho-Elk-1 antibodies (*P-Elk-1*). *C*, the same blot was sequentially analyzed by Western blotting to determine MAPK activation using anti-phospho-ERK antibodies (*P-Erk*) and was probed with anti-ERK1/2 antibodies (*Erk2*) as a loading control. Three independent experiments produced similar results.

ionomycin (1 μM) used, it appeared that the phospho-Elk-1 level was slightly decreased in Me_2SO-treated control cells (Fig. 5*B*); however, no suppression of c-Fos expression could be detected in the same experiment (Fig. 5*A*). 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. 6*A*), Elk-1 phosphorylation (Fig. 6*B*), 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

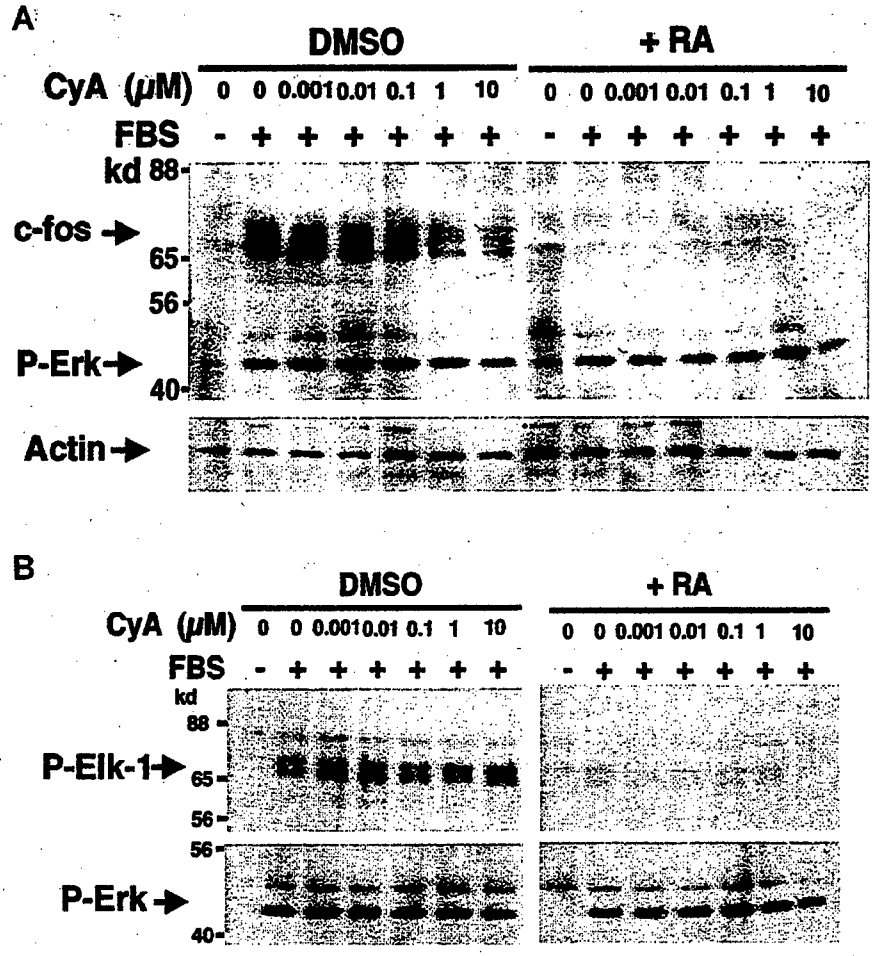

FIG. 6. **Effects of cyclosporin A on serum-stimulated c-Fos expression and MAPK activation.** F9 cells were cultured for 4 days with 1 μM retinoic acid (*RA*) or Me_2SO (*DMSO*) solvent control. The PP2B inhibitor cyclosporin A (*CyA*) was added at 1–10 μM for 60 min prior to stimulation with 15% FBS. *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.

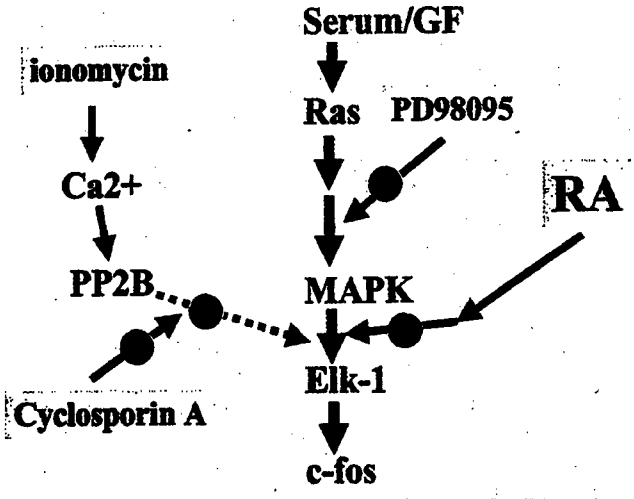

FIG. 7. **Disassociation of c-Fos expression and MAPK activation in retinoic acid-induced F9 cell differentiation.** Shown is a schematic illustration of the effect of retinoic acid (*RA*), PD98095, ionomycin, and cyclosporin A on MAPK activation, Elk-1 phosphorylation, and c-Fos expression. Retinoic 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.

and Elk-1 phosphorylation/activation when transfected into mammalian cells, although the mechanism is yet unknown (67). A third example is α-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 α-synuclein prominently attenuates Elk-1 phosphorylation without inhibiting MAPK (68). α-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. 3*A*) (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

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) and the calcium ionophore and potential PP2B activator (ionomycin) have no effect on c-Fos expression with or without retinoic acid treatment of F9 cells. To modulate 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 to these agents (56–58, 67). It is possible that PP2B is not expressed in F9 cells, and this possibility remains to be examined.

Alternatively, retinoic acid treatment may inhibit phosphorylation of Elk-1 by MAPK, such as preventing the productive association between Elk-1 and MAPK, as in the case of α-synuclein (68), blocking nuclear entry of activated MAPK, sequestration of Elk-1 from being phosphorylated by activated MAPK. We are currently investigating these possibilities.

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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Disabled-2 Mediates c-Fos Suppression and the Cell Growth Regulatory Activity of Retinoic Acid in Embryonic Carcinoma Cells*

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F9 embryonic stem cell-like teratocarcinoma cells are widely used to study early embryonic development and cell differentiation. The cells can be induced by retinoic acid to undergo endodermal differentiation. The retinoic acid-induced differentiation accompanies cell growth suppression, and thus, F9 cells are also often used as a model for analysis of retinoic acid biological activity. We have recently shown that MAPK activation and c-Fos expression are uncoupled in F9 cells upon retinoic acid-induced endodermal differentiation. The expression of the candidate tumor suppressor Disabled-2 is induced and correlates with cell growth suppression in F9 cells. We were not able to establish stable Disabled-2 expression by cDNA transfection in F9 cells without induction of spontaneous cell differentiation. Transient transfection of Dab2 by adenoviral vector nevertheless suppresses Elk-1 phosphorylation, c-Fos expression, and cell growth. In PA-1, another teratocarcinoma cell line of human origin that has no or very low levels of Disabled-2, retinoic acid fails to induce Disabled-2, correlating with a lack of growth suppression, although PA-1 is responsive to retinoic acid in morphological change. Transfection and expression of Disabled-2 in PA-1 cells mimic the effects of retinoic acid on growth suppression; the Disabled-2-expressing cells reach a much lower saturation density, and serum-stimulated c-Fos expression is greatly suppressed and disassociated from MAPK activation. Thus, Dab2 is one of the principal genes induced by retinoic acid involved in cell growth suppression, and expression of Dab2 alone is sufficient for uncoupling of MAPK activation and c-Fos expression. Resistance to retinoic acid regulation in PA-1 cells likely results from defects in retinoic acid up-regulation of Dab2 expression.

Disabled-2 (*DAB2*[1] for the human gene and Dab2 for the protein and gene in other species) is one of the two mammalian orthologs of the *Drosophila* Disabled that was identified as one of the proteins genetically interacting with Abl kinase in fly neuron development (1, 2). The three spliced forms (p96, p93, and p67) of murine Dab2 cDNA were first isolated as mitogen-responsive phosphoproteins functioning in the CSF-1 signal transduction pathway in macrophages (2). DAB2 is thought to be a tumor suppressor in ovarian cancer (3–6). Its expression is lost or greatly diminished in 85% of the breast and ovarian cancers analyzed (5), and forced re-expression of Dab2 suppresses cell growth and tumorigenicity (4, 6, 7). Gene deletions have been found to account for the loss of DAB2 expression in a small percent of tumors.[2]

In vertebrates, retinoic acid plays a role in inducing cell lineage in early embryonic development, and defects in retinoic acid metabolism or exposure may result in abnormal development (9, 10). The GATA transcription factors are believed to serve as mediators of retinoic acid in the induction of the heart, gut, and hematopoietic systems during development (9–13). Retinoic acid induces gene expression and differentiation in many cell types in culture and exhibits growth suppressive activity in a wide spectrum of tumor cells. Furthermore, retinoic acid has been used successfully to treat leukemia and has been explored for use in treating other malignancies (14–16). In *in vitro* studies of cultured tumor cells, retinoic acid suppresses cyclin D induction and saturation cell density but does not affect log phase cell growth (17, 18). One of the several possible mechanisms postulated for the effect of retinoic acid on cell growth inhibition is the suppression of AP-1 activity (19, 20), which is the target of activation of the Ras/MEK (kinase for MAPK or Erk)/MAPK pathway by many mitogens. Retinoic acid also induces the transforming growth factor-β pathway, another route for tumor/growth suppression in some systems (21). The action of retinoic acid is mediated through nuclear receptors that in turn modulate gene expression (9, 22). Although some of the direct transcriptional targets of retinoic acid are known, such as the GATA factors (11) and laminin (23), the principal retinoic acid-controlled growth regulator(s) has yet to be identified, and the mechanisms for retinoic acid regulation and resistance are as yet not fully understood. One of the remarkable changes in cell properties identified recently is that retinoic acid-induced differentiation of F9 cells accompanies the uncoupling of MAPK activation and c-Fos expression (24), although the mediators of this retinoic acid-induced alteration have not been identified.

In addition, some tumor cells develop resistance to growth suppression by retinoic acid (25). Loss of retinoic acid receptors accounts for some cases, but other unidentified mechanisms must exist (19, 25, 26). In this study using F9 (retinoic acid-

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[1] The abbreviations used are: Dab2, Disabled-2; FBS, fetal bovine serum; MAPK (Erk), mitogen-activated protein kinase (Erk, extracellular-signal regulated kinase); DMEM, Dulbecco's modified Eagle's medium; MOPS, 4-morpholinepropanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

[2] Z. Fazili, Z. Sheng, W. Sun, C. Cohen, L. E. Mendez, I. R. Horowitz, A. K. Godwin, and X. X. Xu, submitted for publication.

sensitive) and PA-1 (retinoic acid-resistant) teratocarcinoma cell lines, we identified the candidate tumor suppressor Dab2 as a retinoic acid-inducible gene in F9 cells but not in PA-1 cells. Dab2 was found to mediate the retinoic acid effect on cell growth inhibition by suppressing c-Fos induction without altering MAPK activation. Transfection/expression of Dab2 is sufficient for cell growth suppression, suggesting that Dab2 is the major mediator of retinoic acid in cell growth suppression. Moreover, the failure or inability to induce Dab2 may be a mechanism for the resistance of tumor cells to retinoic acid in growth suppression.

EXPERIMENTAL PROCEDURES

Materials—Retinoic acid (all-*trans*-, 9-*cis*-retinoic acid) and β-carotene were purchased from Sigma. Tissue culture supplies were obtained from Fisher. DMEM medium was purchased from Mediatech (Herndon, VA); fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA); TRIzol reagent, 100× antibiotic-antimycotic solution, LipofectAMINE, and serum-free Opti-MEM I medium were purchased from Life Technologies, Inc.; the ECL Super-Signal West Dura extended duration substrate immunodetection reagents were purchased from Pierce; Hybrisol I hybridization solution came from Intergen (Purchase, NY); positively charged nylon membranes were from Roche Molecular Biochemicals; [α-^{32}P]dCTP was from PerkinElmer Life Sciences. All other general chemicals and supplies including Me_2SO, ethanol, isopropanol, and agarose were from Sigma or Fisher and were reagent grade or higher.

Cell Culture—F9 mouse teratocarcinoma and PA-1 human teratocarcinoma cells were purchased from American Type Culture Collection (ATCC). The PA-1 cells were cultured in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution. F9 cells were cultured on gelatin-coated tissue culture plates in DMEM containing 10% heat-inactivated FBS and 1× antibiotic-antimycotic solution. The plates were coated with an autoclaved 0.1% gelatin solution overnight at 4 °C, then washed three times with phosphate-buffered saline before use. Retinoids were added to cells from a 1 mM stock solution in Me_2SO. If it is not specifically stated, all-*trans*-retinoic acid was used. Control cultures contained an equal volume of Me_2SO alone. Usually, retinoic acid was added 24 h after plating of cells. Cell growth was determined by either triplicate counting with a hemacytometer or measured using the MTT assay (Promega). The results of MTT assay agreed well with those from cell counting.

Antibodies and Western Blot Analysis—Anti-Dab2 antibodies were characterized as previously described (2, 5, 6, 27). Anti-Dab2 (p96) monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY); anti-c-Fos came from Santa Cruz Technology; anti-actin came from Sigma; anti-Erk1/2 and anti-phospho-Erk1/2 came from Cell Signaling Technology, Inc. (Beverly, MA). Immunoblotting was performed according to standard procedures, as described previously (5, 6, 27). After confirmation of antibody selectivity, in some cases two or more antibodies were used simultaneously in an incubation to detect various molecular weight proteins.

Northern Blot Analysis—Total RNA was isolated from cell monolayers according to the TRIzol method (Life Technologies, Inc.). RNA was separated on 1% agarose gel containing 7% formaldehyde and 20 mM MOPS buffer, transferred to positive-charged nylon membranes using 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) buffer, and fixed by baking. DNA probes were labeled with [α-^{32}P]dCTP using a random prime labeling kit (Amersham Pharmacia Biotech). The hybridization and Northern blotting followed standard procedures as described previously (2, 5).

Cell Transfection—The full-length human DAB2 (28) or murine Dab2 (2) cDNA was inserted into the pcDNA/zeo (Invitrogen, La Jolla, CA) or pMT-CB6+ eukaryotic expression vectors. Plasmid DNA was purified using Qiagen Maxiprep columns. For transfection, 2 μg of Dab2 or vector plasmid DNA were mixed with 20 μl LipofectAMINE in 1 ml of Opti-MEM and added to PA-1 or F9 cells for 16 h. F9 cells were transfected with mouse Dab2 cDNA, and PA-1 cells were transfected with human Dab2 cDNA. The transfection medium was removed, and fresh DMEM containing 10% FBS was added. After 12 h, transfected cells were cultured in DMEM containing 10% FBS and 300 ng/ml zeomycin for selection of pcDNA/zeo vector or 400 μg/ml G418 for selection of pMT-CB6+ vector. This selection medium was changed every 2 days, and after 10–12 days cloning rings were used to isolate positive clones. Cultures were further expanded and examined for Dab2 expression by Western blotting.

F9 cells were also transfected with metallothionein promoter-regulated mouse Dab2 construct in pMT-CB6+ vector, and green fluorescent protein in pMT-CB6+ vector was used as a control. To induce expression, 0.1 mM $ZnSO_4$ was added to the medium for 24–72 h.

Cell Cycle Analysis—Cell monolayers were released from plates with 0.25% trypsin, 0.1% EDTA and collected by centrifugation. The cells were then fixed in 70% ethanol at 4 °C, pelleted, and re-suspended in 50 μg/ml propidium iodide in phosphate-buffered saline for 30 min at 4 °C. The stained cells were analyzed by flow cytometry performed on a FACScan equipped with argon-ion laser and analyzed by Cell Quest software (Becton Dickinson).

Transient Transfection of Dab2 Using Adenoviral Approach—Replication-deficient adenovirus expressing Dab2 p96 or p67 spliced forms or β-galactosidase were produced, purified, and titrated as described previously (6). For transfection of F9 or PA-1 cells, 100 multiplicity plaque-forming units of adenovirus were added to the cells in medium with low serum (1% FBS) for 4 h. The cells were then used for further experimental manipulation. Under these conditions, more than 90% of the F9 cells expressed the transfected cDNA, as estimated using adenovirus-expressing β-galactosidase.

RESULTS

Induction of Disabled-2 Expression by Retinoic Acid—Dab2, a candidate tumor suppressor, is lost in a wide spectrum of tumor tissues and cultured carcinoma cells (5). To evaluate mechanisms for its loss, we examined potential factors that might affect Dab2 expression. Dab1, the human ortholog that is mainly expressed in brain, can be induced by retinoic acid in the embryonic P19 carcinoma cell line (29) and by thyroid hormone (T3 and T4) (30). Thus we investigated and found that Dab2 can also be induced by retinoic acid in the mouse embryonic teratocarcinoma F9 cell line, which is widely used as a model for studying effects of retinoic acid in gene transcription and cell differentiation. Another recent report also confirmed the ability of retinoic acid to induce Dab2 expression (31).

We found that retinoic acid induces expression of both of the two variably spliced forms of Dab2, p96 and p67 (2), in F9 cells. The effect is time (Fig. 1*A*)- and dose-dependent (Fig. 1*B*). High levels of Dab2 protein were induced after treatment with retinoic acid for 4 days, and as little as 10^{-8} M retinoic acid stimulated Dab2 protein expression. Retinoic acid treatment caused greater induction of the p67 form of Dab2, which differs from the expression pattern of Dab2 isoforms found in other cells in which p96 is generally the major or only isoform (2, 5). The induction of Dab2 by retinoic acid occurs at the transcriptional level, because the Dab2 message RNA is induced in a similar magnitude as the protein (Fig. 1*C*). Withdrawal of retinoic acid 4 days after induction did not reverse or decrease Dab2 protein levels (Fig. 1*D*), and even a month after retinoic acid removal F9 cells continued to express Dab2 (not shown). These results correlate Dab2 expression with the irreversible endoderm differentiation of F9 cells by retinoic acid treatment. Among the retinoids tested, all-*trans*-retinoid acid is the most potent in the induction of Dab2 (Fig. 1*E*). 9-*cis*-Retinoic acid can induce Dab2 expression in F9 cells, but the required dosage is about 100 times more than that of all-*trans*-retinoic acid, and *N*-(4-hydroxylphenyl)retinamide (fenretinide or 4-HPR) and β-carotene (vitamin A) have no detectable activity (Fig. 1*E*).

In the PA-1 teratocarcinoma cell line, however, retinoic acid treatment for 4 days did not induce Dab2 expression (Fig. 2*A*). PA-1 cells were derived from a human ovarian germ cell tumor (32) and are resistant to growth suppression by retinoic acid (19, 33, 34), in contrast to F9 cells. Longer duration of treatment with 1 μM retinoic acid for 2 weeks still failed to induce Dab2 expression in PA-1 cells (data not shown). The lack of Dab2 induction occurs at the transcriptional level, because no changes in DAB2 mRNA were observed (Fig. 2*B*). RNA from ES2 cells, a Dab2-positive ovarian cancer cell line (5), was used as a positive control.

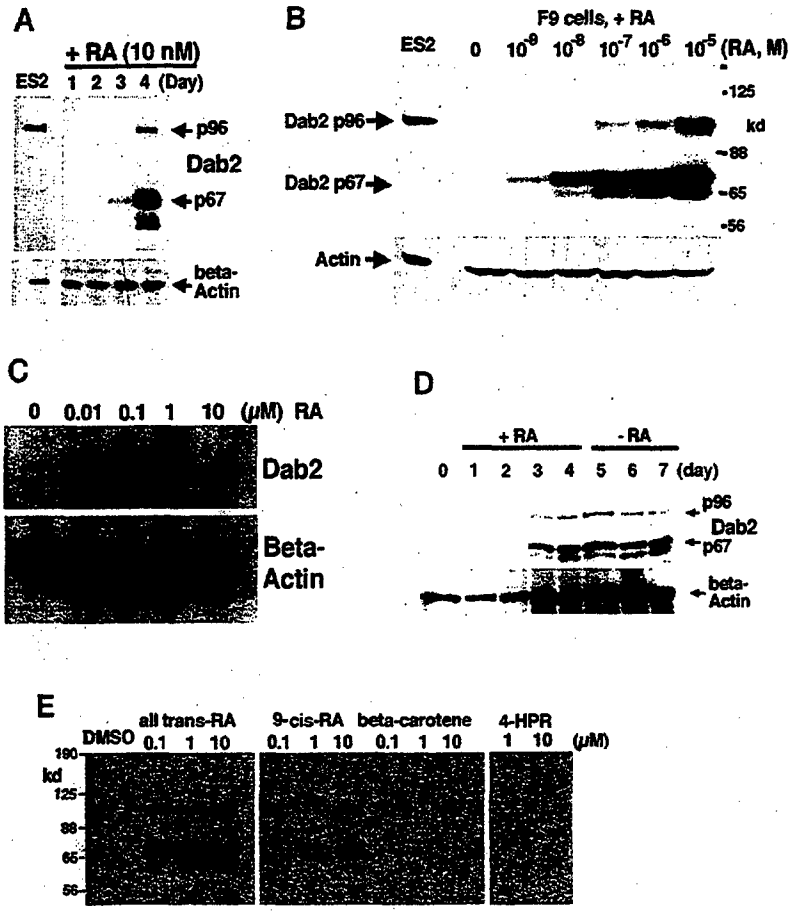

FIG. 1. **Induction of Dab2 expression in F9 cells.** *A*, time course of Dab2 protein induction. F9 cells were seeded on 35-mm plates (5×10^4 cells/dish) on day 0. After incubation for 3 h, all-*trans*-retinoic acid (*RA*, 10 nM) was added. Cell lysates were prepared on days 1, 2, 3, and 4 and used to measure Dab2 protein level by Western blot. β-Actin was determined as a protein loading control, and a lysate of ES2 cells was used as a Dab2-positive control. *B*, dose dependence of retinoic acid induction. Dab2 protein expression was determined by Western blot in F9 cells incubated with increasing concentrations of retinoic acid for 4 days. *M*, molarity. *C*, retinoic acid induction of Dab2 mRNA. Approximately 1×10^6 F9 cells were plated on 100-mm plates and incubated with retinoic acid for the indicated days. Total RNA was isolated and analyzed for Dab2 mRNA levels by Northern blotting. *D*, irreversible retinoic acid-induced Dab2 expression. F9 cells were first stimulated with 1 μM retinoic acid for 4 days. On day 4, retinoic acid was removed, and the cells were washed, replated on new culture dishes, and cultured for additional days without retinoic acid. At the indicated times, cells were lysed and analyzed for Dab2 protein by Western blotting. β-Actin protein level was determined as a loading control. *E*, activity of retinoids in the induction of Dab2. F9 cells were treated with Me_2SO (*DMSO*) solvent alone or with 0.1, 1, or 10 μM all-*trans*-retinoic acid, 9-*cis*-retinoic acid, fenretinide (4-HPR), or β-carotene for 4 days. The cell lysate were used to determined Dab2 level by Western blotting.

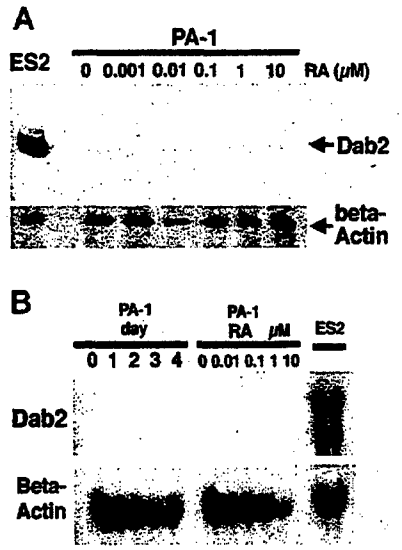

FIG. 2. **Lack of effect of retinoic acid (*RA*) on Dab2 expression in PA-1 cells.** PA-1 cells were cultured and analyzed for Dab2 expression exactly as described for F9 cells in Fig. 1. *A*, retinoic acid effect on Dab2 protein expression. Dab2 protein expression was determined by Western blot in PA-1 cells incubated with increasing concentrations of retinoic acid for 4 days. *B*, effect of retinoic acid on Dab2 mRNA levels. Total RNA from ES2 cells was used as a positive control.

Retinoic Acid Induces Cell Growth Suppression in F9 but Not in PA-1 Teratocarcinoma Cells and Induces Morphological Changes in Both Cell Lines—In parallel experiments, retinoic acid inhibited the growth of F9 cells in a time (Fig. 3*A*)- and dose-dependent manner (Fig. 3*C*) and also caused morphological changes of the cells in culture (Fig. 3*D*). Suppression of cell growth correlated with the induction of Dab2 expression since both occurred at day 3 after treatment with retinoic acid. F9 cells treated with retinoic acid for 4 days were well separated and dispersed compared with non-treated cells, which appeared tightly packed and physically connected. In contrast, retinoic acid had no effect on PA-1 cell growth (Fig. 3, *B* and *C*), although a morphological change was seen, in agreement with previous reports (19, 33, 34). In PA-1 cells treated with 1 μM retinoic acid for 4 days (Fig. 3*E*), cells appear to be less elongated and the nuclei more pronounced. Thus, retinoic acid induces morphological changes and cell growth suppression in F9 cells and induces morphological changes but no growth suppression in PA-1 cells. Resistance to retinoic acid-induced growth suppression, therefore, correlates with a lack of Dab2 induction.

Transfection and Expression of Dab2 Mimics the Effect of Retinoic Acid on Cell Growth—To examine the effect of Dab2 on cell growth and morphology, a Dab2 expression construct was transfected into both F9 and PA-1 cells. In F9 cells transfected with Dab2, only three G418-resistant colonies were selected compared with 64 resistant colonies of vector controls in parallel transfection. After expansion of the three Dab2-transfected clones, none were found to express the Dab2 protein as detected by Western blotting. We then transfected F9 cells with mouse Dab2 p96 construct under the control of the metallothionein promoter (pMT-CB6+ vector). In 48 clones selected for analysis, at least 6 clones appear to express the Dab2 p96 protein (Fig. 4*A*). However, we have also observed that the $ZnSO_4$-induced F9 cells undergo differentiation without retinoic acid; the cells also express the p67 form of Dab2 (although these were transfected with the p96 form of Dab2), and the cells also express GATA-4, GATA-6, collagen IV α2, and laminin, which are markers for differentiated endoderm cells. Thus, we

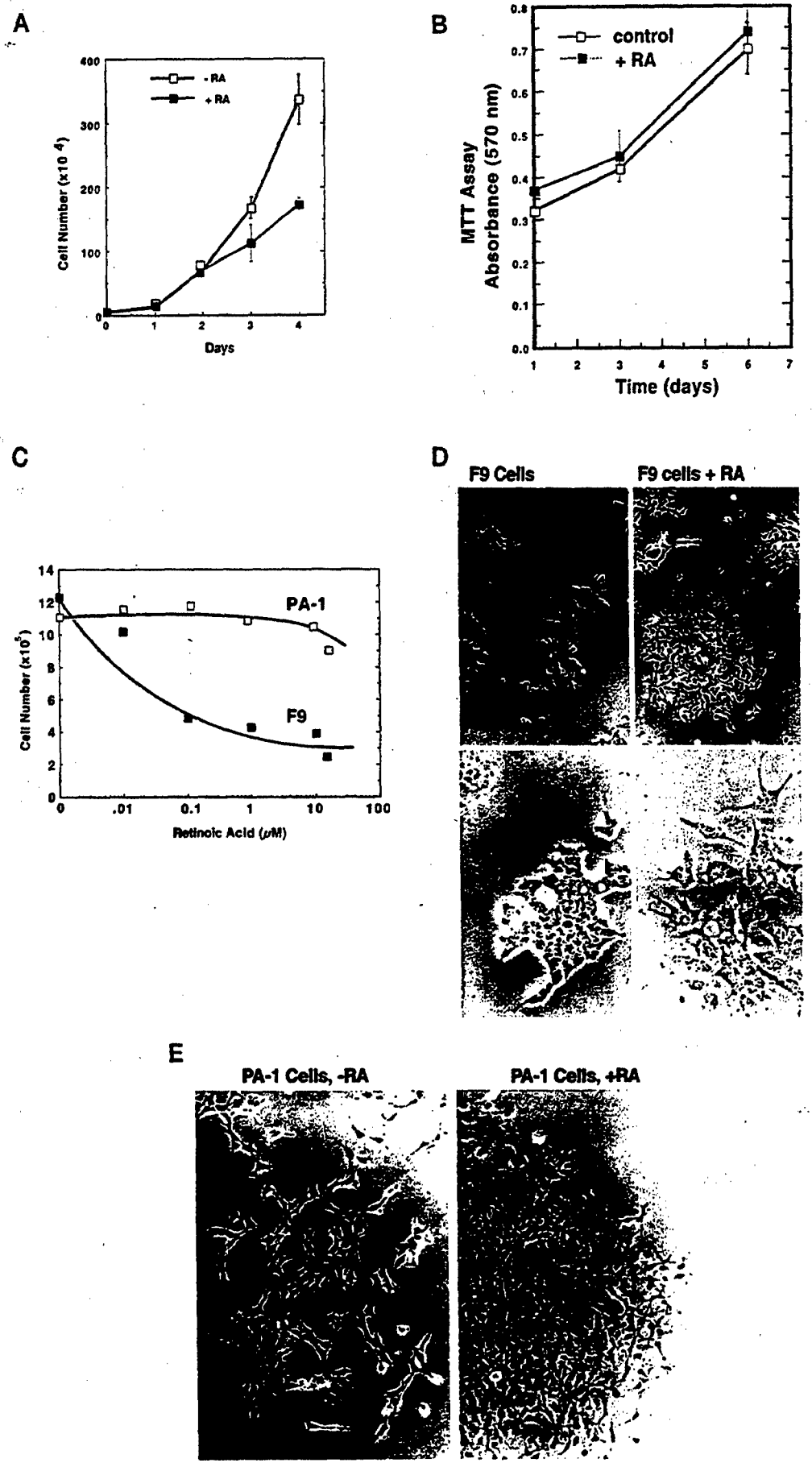

FIG. 3. **Effect of retinoic acid (*RA*) on F9 and PA-1 cell growth and morphology.** F9 (*A*) or PA-1 (*B*) cells were plated at 5×10^4 cells/well on day 0. After allowing the cells to attach for 3 h, retinoic acid (1 μM) was added. Cell number was determined by cell counting, and the MTT assay

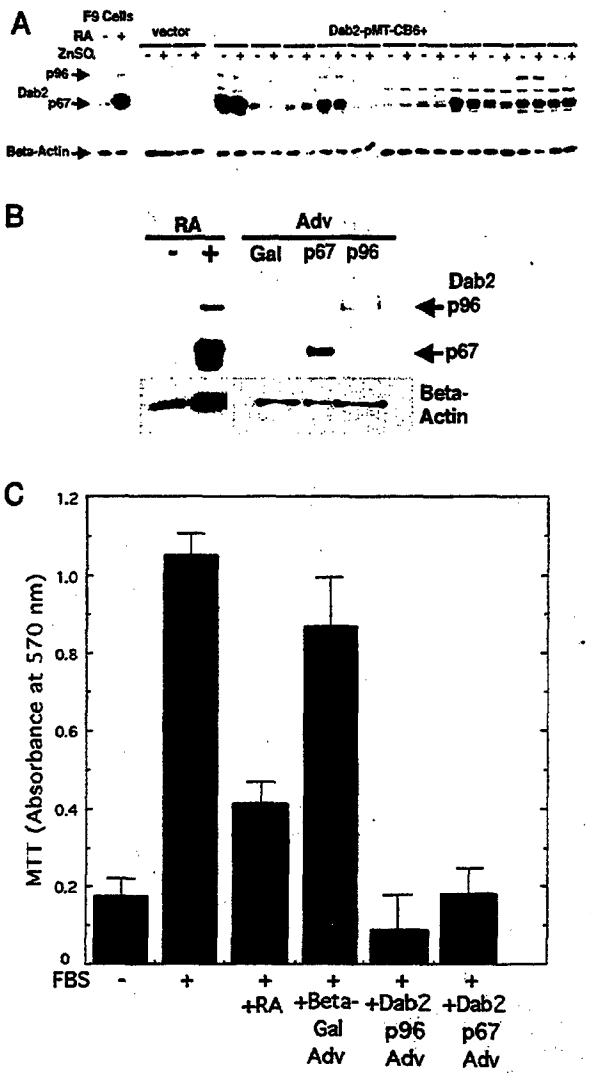

FIG. 4. **Transfection of F9 cells.** *A*, clones of F9 cells transfected with Dab2 in pMT-CB6+ and induced with 0.1 mM $ZnSO_4$ for 24 h were analyzed for Dab2 expression by Western blotting. *B*, F9 cells transfected with adenovirus (*Adv*) carrying Dab2 p96 or p67 isoforms for 4 days were analyzed for Dab2 expression by Western blotting. *C*, the cell numbers were determined using MTT assay in F9 cells treated for 4 days with or without retinoic acid (1 μM), with adenovirus expressing β-galactosidase (*Gal*) or Dab2 p67 or p96. *RA*, retinoic acid.

conclude that it is not possible to obtain stable Dab2-expressing F9 cells without also inducing spontaneous retinoic acid-independent differentiation. However, we are able to transiently express Dab2 by adenoviral approach (Fig. 4*B*) without inducing differentiation of the F9 cells, as judged by the lack of expression of the p67 spliced form of Dab2, GATA-4, and GATA-6. Dab2 expression by the adenoviral approach suppresses F9 cell growth (Fig. 4*C*), suggesting that retinoic acid-induced Dab2 expression is responsible for the cell growth inhibitory activity of retinoic acid in F9 cells.

There are undoubtedly many differences in the genetic background and properties of mouse F9 and human PA-1 teratocarcinoma cells, although both cell lines have some properties of embryonic stem cells. Although both are undifferentiated and multipotent, PA-1 cells synthesize collagen IV and laminin (32–34), unlike F9 cells, which do not express collagen IV and laminin before retinoic acid-induced differentiation (26). After transfection of PA-1 cells with a Dab2 expression construct, 16 colonies were selected compared with 54 colonies from vector-transfected controls. All of the Dab2-transfected colonies developed much more slowly (estimated to be 20-fold less based on cell number) than colonies from vector-transfected controls, as shown in Fig. 5*A* for a typical example of a G418-selected colony. Under identical culture conditions, the Dab2-transfected cells appeared well separated from each other within a colony, whereas the vector-transfected control cells in a colony were aggregated and indistinguishable from parental cells. In an earlier passage with a cell number of about 1×10^5 cells/colony, Dab2 expression was detected. Only the p96 form of Dab2 but not the p67 form was expressed, suggesting that Dab2 expression was the result of cDNA transfection and not spontaneous differentiation. However, as cultures were expanded, the morphological difference diminished, and Dab2 expression was gradually lost in most of the clones. For three colonies, Dab2 expression remained after several passages, and the morphological changes, although not as obvious as for the cells in earlier passages, were still apparent compared with vector-transfected cells (Fig. 5*B*). Additionally, these Dab2-expressing cells exhibited a reduced growth rate compared with vector-transfected controls (Fig. 5*C*). The ability to form colonies on agar plates was suppressed upon Dab2 expression (Fig. 5*D*). Therefore, transfection experiments indicate that expression of Dab2 suppresses cell proliferation and anchorage-independent colony formation and alters cell-cell adhesion.

Moreover, these changes correlate well with alterations in the cell cycle. Under identical culture conditions as described above, the two transfected PA-1 clones with detectable Dab2 expression (clones 9 and 13) had an increase in the percentage of cells in G_1 and a corresponding decrease of cells in S phase compared with vector-transfected or parental cells (Table I). Thus, Dab2 inhibits cell growth by suppressing G_1 phase progression, which is similar to the effect of retinoic acid on the cell cycle (17, 18, 26).

Dab2 Transfection and Expression Inhibits Serum-stimulated c-Fos Expression and Uncoupling from MAPK Activation—We next examined the effect of Dab2 on end points of the mitogenic signaling pathway compared with the effect of retinoic acid on the signaling properties of F9 cells. Expression of Dab2 in breast cancer cells results in the disassociation of MAPK activation and c-Fos expression (35). It is thought that retinoic acid reduces cell growth by suppressing AP-1 activity of the Jun/Fos transcription complex (19, 20) as does Dab2 (36). In F9 cells, we have found that retinoic acid-induced differentiation results in a much weaker c-Fos induction by serum, although MAPK activation is not affected (Fig. 6*A*). By adenovirus transfection of Dab2, without induction of endoderm differentiation, the Elk-1 phosphorylation and the c-Fos expression are inhibited (Fig. 6*B*). Thus, expression of Dab2 alone is sufficient to alter the signaling in F9 cells.

Whereas c-Fos induction and MAPK activation in PA-1 cells were not altered by retinoic acid treatment (Fig. 6*C*), we found that the expression of c-Fos was greatly reduced after serum stimulation in PA-1 cells expressing Dab2 (Fig. 6*C*). Remarkably, the MAPK activation in these cells was not affected by Dab2 expression. Thus, we conclude that Dab2 can suppress cell growth by inhibiting c-Fos expression as a result of uncou-

with the absorbance at 570 nm was reported. All experiments were performed in triplicate. *Bars*, S.E. *C*, F9 (*filled squares*) and PA-1 (*open squares*) cells were incubated with increasing concentrations of retinoic acid for 4 days. Cell numbers/well were determined by counting using a hemacytometer. To determine the effect of retinoic acid on cell morphology, F9 (*D*) and PA-1 (*E*) were incubated with 1 μM retinoic acid or Me_2SO vehicle for 4 days. *Top panels* in *D*, ×100 magnification; *bottom panels*, ×400 magnification.

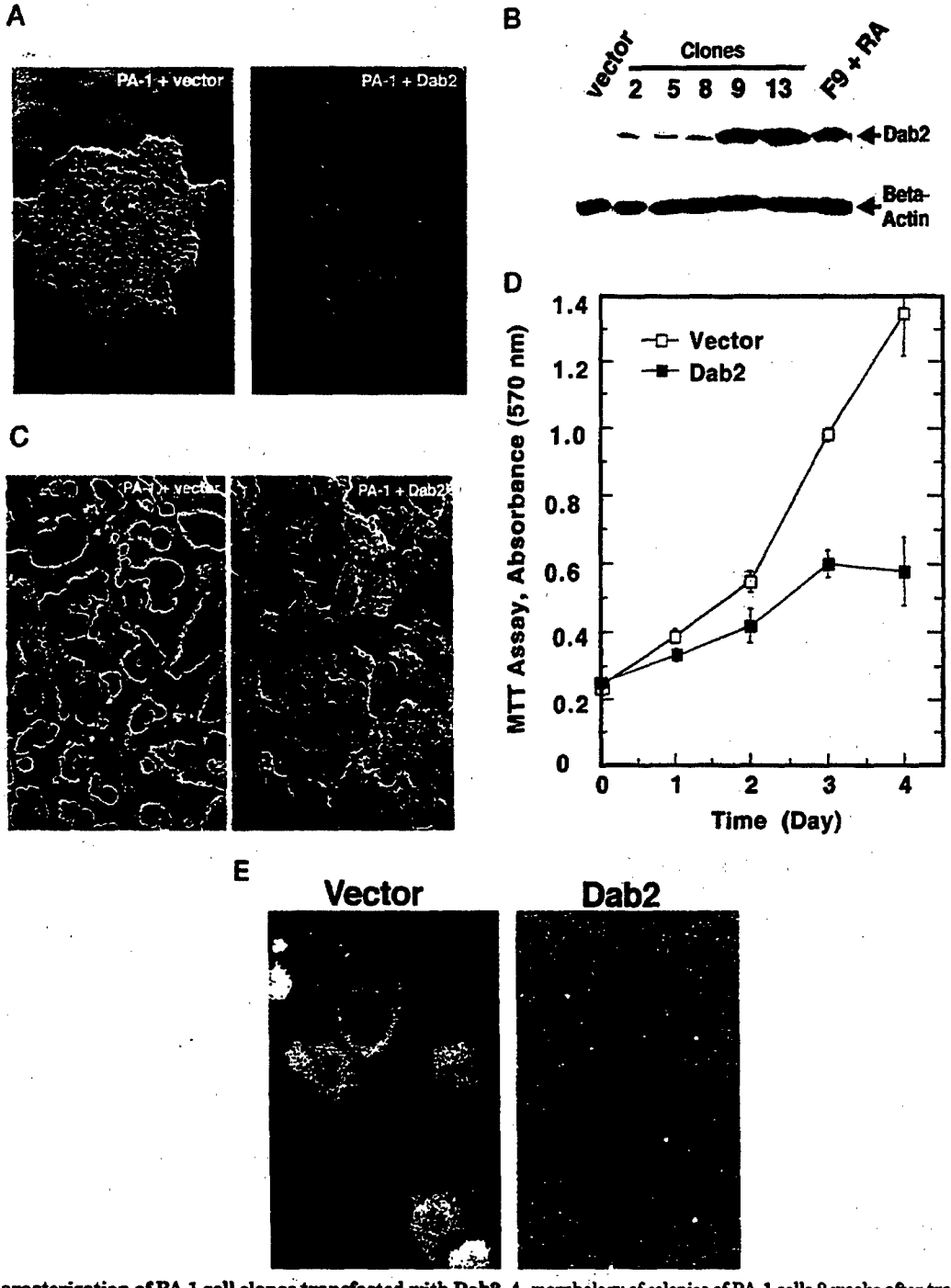

FIG. 5. **Characterization of PA-1 cell clones transfected with Dab2.** *A*, morphology of colonies of PA-1 cells 2 weeks after transfection with either vector or Dab2 and selection using G418. *B*, morphology of vector- and Dab2-transfected PA-1 cells after expansion of cell cultures. Both *A* and *B* were photographed at 100× magnification. *RA*, retinoic acid. *C*, expression of Dab2 in transfected clones of PA-1 cells. The cells from each clone were analyzed by Western blot for Dab2 expression and β-actin as protein loading controls. *D*, growth curve of vector or Dab2 construct-transfected PA-1 cells. Clones of vector or Dab2 transfected PA-1 cells were selected with G418 and characterized for Dab2 expression by Western blotting. The growth rate of a representative clone was shown. An equal number (5×10^3 cells/well) of PA-1 cloned cells transfected with vector (*open squares*) or Dab2 (*filled squares*) were plated on 96-well plates. Cell numbers were determined every day by MTT assay and are expressed as the mean absorbance of triplicates at 570 nm; *bars*, S.E. *E*, Dab2 expression suppresses colony growth by PA-1 cells on soft agar. PA-1 cells (1×10^5 cells/35-mm well) transfected with vector or Dab2 were grown on agar plates for 4 weeks. A representative field of the plates is shown.

pling from MAPK activation, and expression of Dab2 mimics retinoic acid in inhibiting cell growth. Expression of Dab2 either by retinoic acid induction in F9 cells or transfection in PA-1 cells results in cell growth suppression and disassociation of c-Fos expression from MAPK activation.

DISCUSSION

Both F9 and PA-1 cells are well characterized teratocarcinoma lines derived from tumors of gonads (testes and ovary). 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, 26, 31, 37). The PA-1 line also shares similar properties to embryonic cells (19, 32–34). Although PA-1 cells can be differentiated or affected in morphology by retinoic acid (19, 33, 34), they are resistant to the growth suppressive activity of retinoic acid. Herein we have demonstrated that Dab2 is induced by retinoic acid in the F9 mouse teratocarcinoma cells

TABLE I
Cell flow cytometry analysis

PA-1 cells were transfected with vector or a human Dab2 expression construct and selected in DMEM containing 10% FBS and zeomycin. Two clones (Clone 9 and 13) were isolated that stably expressed Dab2 after expansion of the cultures. The parental, vector-transfected, and Dab2-transfected clones were analyzed for cell cycle distribution by flow cytometry, as described under "Experimental Procedures."

Cell	G_1	S	G_2 + M
		% total cells	
PA-1	48	27	25
Vector	51	24	25
Dab2 Clone 9	75	8	17
Dab2 Clone 13	66	8	26

but not in retinoic acid-resistant PA-1 cells. Moreover, Dab2 expression suppresses c-Fos expression by uncoupling it from MAPK activation and accounts for or contributes to the growth suppressive activity of retinoic acid in F9 cells. At least two additional proteins, KSR (kinase suppressor of Ras) (38) and Gab2 (39), have been reported to uncouple MAPK activation and c-Fos expression. This regulatory step adds additional complexity and flexibility in the Ras pathway.

Although the mechanism for growth suppression by retinoic acid is not yet certain, several possibilities have been investigated, including the induction of the transforming growth factor-β pathway (21) and inhibition of AP-1 activity by competition of the cofactor CBP (40). Here, we propose another pathway as follows. Retinoic acid inhibits AP-1 activity by reducing serum-stimulated c-Fos induction in a Dab2-dependent manner (Fig. 6). Dab2 binds the adapter protein Grb2 (growth factor receptor binding protein 2) and may affect Ras signaling (27) and suppresses AP-1 activity (37). We observe here that treatment of F9 cells with retinoic acid results in a much weaker induction of c-Fos by serum as a result of uncoupling it from MAPK activation, correlating with the expression of Dab2 (24). Remarkably, expression of Dab2 alone can inhibit serum-stimulated c-Fos expression by uncoupling from MAPK activation in transfected breast cancer cells (35), in F9 cells transfected with adenovirus carrying Dab2 cDNA (Fig. 6*B*), and in Dab2-transfected PA-1 teratocarcinoma cells (Fig. 6*C*). Thus, the suppression of F9 cell growth and c-Fos expression upon retinoic acid treatment is largely mediated by Dab2.

We were able to stably transfect and express Dab2 in PA-1 but not F9 cells. We reason that the presence of some additional partners such as collagen IV and laminin in PA-1 cells may have assisted for the tolerance of Dab2 expression in the cells. Additionally, during the transfection and selection of Dab2-expressing clones, the F9 cells often undergo retinoic acid-independent differentiation. Nevertheless, we were able to transiently express Dab2 without inducing endodermal differentiation of F9 cells using an adenoviral vector. Consistently, expression of Dab2 alone appears to be highly growth-suppressive (Fig. 4*C*). We have not observed spontaneous expression of Dab2 in PA-1 cells under various culture conditions and experimental procedures, making the PA-1 cell line a good model for analyzing the effect of Dab2 transfection/expression. PA-1 cells transfected with Dab2 appear less adhesive to each other, which leads us to speculate that Dab2 expression affects cell contact. Consistent with this idea, Dab2 has been shown to bind to the intracellular domain of Megalin (41). Megalin, a large glycoprotein that can act as a receptor for multiple extracellular ligands, is believed to function in cell-cell and cell-matrix interaction (42). Dab2 shows 30% sequence homology with *Drosophila* Dab and is 45% identical and 60% homologous to Dab1, the other mammalian ortholog (2, 29). Dab1 functions in controlling the positioning of brain cells (43–46) and serves as an adaptor protein in signaling from the extracellular

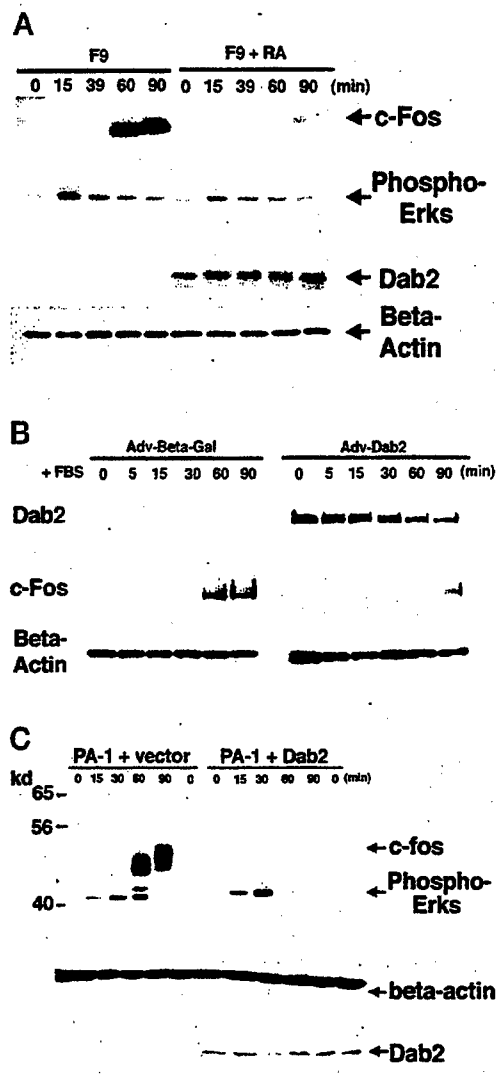

FIG. 6. **Suppression of serum-stimulated c-Fos expression by retinoic acid or Dab2.** *A*, retinoic acid treatment suppresses c-Fos expression in F9 cells. F9 cells were treated with 0.1 μM of retinoic acid or Me_2SO for 4 days. On the last day, the cells were cultured in DMEM with 1% BSA without serum for 18 h. The cells were then stimulated with 10% serum for the indicated times. Cell lysates were analyzed for c-Fos induction and MAPK activation (*Phospho-Erks*) with specific phosphopeptide antibodies by Western blotting. The same blot was also analyzed by Western blotting for β-actin as an indication of protein loading and for Dab2 to verify the induction by retinoic acid (*RA*). *B*, adenovirus (*Adv*)-mediated Dab2 expression suppresses c-Fos expression and Elk-1 phosphorylation in F9 cells. F9 cells were infected with adenovirus carrying β-galactosidase (*Gal*) or Dab2 cDNA and cultured for 2 days. On the last day, the cells were cultured in DMEM with 1% BSA without serum for 18 h. The cells were then stimulated with 10% serum for the indicated times. Cell lysates were analyzed for c-Fos induction, Elk-1 phosphorylation, and MAPK activation by Western blotting. The same blot was also analyzed for β-actin as an indication of protein loading and for Dab2 to verify the expression. *C*, Dab2 transfection and expression suppresses c-Fos expression stimulated by serum in PA-1 cells. Representative clones of PA-1 cells transfected with vector or the Dab2 expression construct were plated on 35-mm dishes and placed in DMEM without serum for 18 h. The cells were then stimulated with 10% serum for the indicated times and lysed, and the lysates were analyzed for c-Fos expression and MAPK activation by Western blotting simultaneously. The same blot was also analyzed by Western for β-actin as an indication of protein loading and for Dab2 to verify the expression.

matrix. This pathway consists of the matrix protein reelin binding to the glycoprotein cell surface receptor, which via Dab1 links to Src family kinases and subsequently further signal transduction (47–49). A similar function is proposed for Dab2 in epithelial

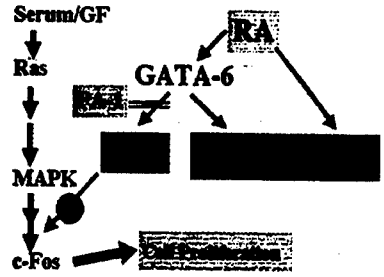

FIG. 7. **Dab2 mediates retinoic acid effects on signal transduction and cell growth in embryonic carcinoma cells.** In embryonic carcinoma cells, retinoic acid (*RA*) induces laminin (*Lam*) expression directly (23) and induces expression of Dab2 and collagen IV (*Col IV*) through the GATA-6 transcription factor (50). Dab2 mediates the effect of retinoic acid on the uncoupling of MAPK activation by serum and growth factors (GF) from stimulation of c-Fos expression and cell growth (24). The induction of Dab2 is speculated to be defective in PA-1 cells.

cell-positioning control such that its loss may contribute to disorganized proliferation found in tumor growth (6). This is consistent with the ability of retinoic acid to induce the synthesis of basement membrane components and induce/maintain cell differentiation, hence organization, of epithelial cells.

Loss or mutations of retinoic acid receptors have been found as the means for tumor cells to acquire resistance to retinoic acid (25, 26), although other mechanisms also exist (9, 14). Because PA-1 cells still respond to retinoic acid with a change in morphology (19, 33, 34), the absence of an effect on growth is likely not because of a loss of functional retinoic acid receptors. Thus, the inability to induce Dab2 expression may be at least one reason for the resistance of PA-1 cells to the growth-suppressive activity of retinoic acid. Though the Dab2 gene is not disrupted in PA-1 cells (data not shown), the cause of Dab2 expression loss in PA-1 cells is not known. The Dab2 promoter lacks known retinoic acid-responsive elements (28), suggesting that retinoic acid indirectly induces Dab2 expression, perhaps through GATA transcription factors (Fig. 7). GATA factors can be induced by retinoic acid (11), and studies of GATA-deficient mouse embryos suggest that GATA-6 is required for Dab2 expression during embryonic development (50). Consistent with these findings, two GATA binding sites are present in the Dab2 promoter (28). Moreover, the transcription of other retinoic acid-inducible genes such as the fibroblast growth factor (51) and the J6 gene (8, 52) also depends on GATA factors.

In conclusion, we found that Dab2 expression is absent or very low in teratocarcinomas such as PA-1 and F9 cell lines. Retinoic acid induces Dab2 expression in F9 but not PA-1 cells (Fig. 7). Furthermore, transfection of Dab2 in F9 and PA-1 cells mimics the retinoic acid-induced suppression on cell growth that occurs in F9 cells. PA-1 cells expressing the transfected Dab2 reach a much lower saturation density, and serum-stimulated c-Fos expression is greatly suppressed as a result of disassociation from MAPK activation. Similar events occur when Dab2 expression is induced in F9 cells, in which the cell growth is suppressed, and c-Fos expression and MAPK activation are uncoupled (24). Thus in F9 cells, Dab2 is one of the principal genes induced by retinoic acid involved in cell growth suppression, and expression of Dab2 alone is sufficient to suppress cell growth to a level that is achieved by retinoic acid treatment. Resistance to retinoic acid regulation in PA-1 cells is at least partially because of deficiency in retinoic acid upregulation of Dab2 expression (Fig. 7).

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