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Raf, but Not MEK or ERK, Is Sufficient for Differentiation of Hippocampal Neuronal Cells

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To elucidate signal transduction pathways leading to neuronal differentiation, we have investigated a conditionally immortalized cell line from rat hippocampal neurons (H19-7) that expresses a temperature-sensitive simian virus 40 large T antigen. Treatment of H19-7 cells with the differentiating agent basic fibroblast growth factor at 39°C, the nonpermissive temperature for T function, resulted in the activation of c-Raf-1, MEK, and mitogen-activated protein (MAP) kinases (ERK1 and -2). To evaluate the role of Raf-1 in neuronal cell differentiation, we stably transfected H19-7 cells with v-*raf* or an oncogenic human Raf-1–estrogen receptor fusion gene (ΔRaf-1:ER). ΔRaf-1:ER transfectants in the presence of estradiol for 1 to 2 days expressed a differentiation phenotype only at the nonpermissive temperature. However, extended exposure of the ΔRaf-1:ER transfectants to estradiol or stable expression of the v-*raf* construct yielded cells that extended processes at the permissive as well as the nonpermissive temperature, suggesting that cells expressing the large T antigen are capable of responding to the Raf differentiation signal. ΔRaf-1:ER, MEK, and MAP kinase activities in the ΔRaf-1:ER cells were elevated constitutively for up to 36 h of estradiol treatment at the permissive temperature. At the nonpermissive temperature, MEK and ERKs were activated to a significantly lesser extent, suggesting that prolonged MAP kinase activation may not be sufficient for differentiation. To test this possibility, H19-7 cells were transfected or microinjected with constitutively activated MEK. The results indicate that prolonged activation of MEK or MAP kinases (ERK1 and -2) is not sufficient for differentiation of H19-7 neuronal cells and raise the possibility that an alternative signaling pathway is required for differentiation of H19-7 cells by Raf.

Biochemical and genetic studies of cells from a wide variety of species have demonstrated that the signals leading to differentiation are highly conserved and that in many cases, the same compounds are involved in both cell growth and transformation. Two general hypotheses have been suggested to explain why common signaling pathways can lead to different endpoints. First, it has been suggested that both the amplitude and the duration of a particular signal can influence the final biological endpoint (reviewed in reference 18). Alternatively, differences in phenotypes have been attributed to differences in specific cellular environments (both internal and external). While it is likely that both factors contribute to the final biological endpoint, it is important to investigate a number of differentiation systems in order to identify their relative contributions.

One major problem, particularly with respect to neuronal cell differentiation, has been the limited availability of appropriate model systems. The best-characterized system to date is the pheochromocytoma cell line PC12, which is derived from a transplantable rat adrenal pheochromocytoma (13). One problem that potentially complicates this system is that the genes leading to immortalization and transformation of PC12 cells have not been identified. However, a major advantage of PC12 cells is that different factors yield different biological endpoints. Epidermal growth factor (EGF) treatment of PC12 cells induces mitogenesis, nerve growth factor whereas or fibroblast growth factor (FGF) treatment induces differentiation to a phenotype with characteristics of sympathetic neurons. The induction of differentiation by nerve growth factor has been correlated with prolonged activation of mitogen-activated protein kinases (MAPKs; ERK1 and -2), and microinjection of constitutively activated MEK (MAPKK) (6) or MAPK (10) has been reported to induce PC12 cell differentiation (6). In addition, it has been shown that Ras and Raf-1 are also able to differentiate PC12 cells (2, 22). Interestingly, v-*raf*-mediated differentiation of PC12 cells was reported to occur under conditions in which MAPK activation was not detected (35, 36). These results led to the model that prolonged MAPK activation is the key mediator of neuronal cell differentiation (reviewed in reference 18) but also raise the possibility of other signaling pathways.

We have generated a cell line (H19-7) derived from early E17 rat hippocampal cells, which provide a neuronal differentiation model for central nervous system neurons (9). These cells have been conditionally immortalized by expression of a temperature-sensitive simian virus 40 large T antigen. Thus, the H19-7 cells offer the advantage of temporary immortalization which can be effectively reversed to yield cells in a more normal state. Like PC12 cells, H19-7 cells respond differentially to EGF and FGF. At the permissive temperature (33°C), EGF treatment induces proliferation; at the nonpermissive temperature (39°C), addition of FGF or phorbol 12,13-dibutyrate (PDBu) but not EGF induces differentiation. The differentiated temperature-sensitive T-antigen-immortalized hippocampal cells do not respond mitogenically to serum, but they

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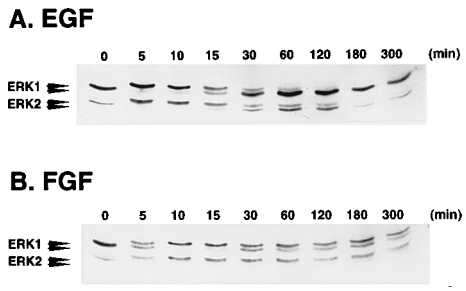

FIG. 1. Time course of ERK phosphorylation following stimulation of H19-7 cells with EGF or bFGF. H19-7 cells were grown in DMEM–10% FBS, then placed in N2 medium, and shifted to 39°C; 48 h later, cells were treated with 10 nM EGF (A) or 10 ng of bFGF per ml (B) for different time intervals. Cell lysates were prepared as described in Materials and Methods, and 25 μg of protein was loaded in each lane and resolved by SDS-PAGE in a 7.5% polyacrylamide gel. The proteins were Western blotted and probed with anti-ERK antibody 283. The hybridized bands were detected by ECL and exposed to X-ray film for autoradiography. ERK1 (44 kDa) and ERK2 (42 kDa) bands are indicated with arrows. These results are representative of three independent experiments.

express neuronal markers such as neurofilaments and brain type II sodium channels and display action potentials (references 8 and 9 and data not shown). Thus, H19-7 cells provide a good model system for testing the role of a variety of signaling molecules in neuronal differentiation.

Analysis of the FGF signaling pathway leading to H19-7 differentiation showed induction of Raf-1 and MEK and a prolonged activation of MAPK. To analyze this pathway further, we transfected these cells with v-*raf* or an oncogenic human Raf-1–estrogen receptor fusion gene (ΔRaf-1:ER). Use of the ΔRaf-1:ER construct enabled us to monitor the immediate events following Raf-1 kinase activation. The results indicate that activated Raf-1 can stimulate neuronal differentiation. However, in this cell system, prolonged MAP kinase activity is not sufficient to induce differentiation.

MATERIALS AND METHODS

Materials. Protein A-Sepharose was purchased from Pharmacia. Fetal bovine serum (FBS), Dulbecco modified Eagle medium (DMEM), Bluo-gal, and Geneticin were purchased from Life Technologies Inc. Hygromycin and estradiol were purchased from Sigma (St. Louis, Mo.). EGF (receptor grade) was purchased from Biomedical Technologies (Stoughton, Mass.). Human basic FGF (bFGF; receptor grade) was purchased from Bachem California (Torrance, Calif.). Myelin basic protein (MBP) was from Sigma. Antiphosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Anti-c-Raf-1 antibody was from Santa Cruz Biotechnology Inc. Monoclonal antibody against the hemagglutinin (HA) epitope was purchased from BabCo (Emeryville, Calif.). Anti-MAPK antiserum (antibody 283) was developed as described previously (4). Antibodies against the high- and middle-molecular-weight neurofilament proteins either were purchased from Sigma (high molecular weight) or were a gift from V. Lee (middle molecular weight). Phosphorylated MAPK-specific antibody was purchased from New England Biolabs, Inc. (Beverly, Mass.). Rat immunoglobulin G (IgG) was purchased from Sigma. Plasmid encoding the kinase-inactive MEK was a gift from G. Johnson. A plasmid, EE-MEK-1 2E-CMV, encoding the mutant MEK-2E protein, and a nonexpressing plasmid, EE-CMV, were generated as previously described (38). Plasmid pLNCΔMEK1:ER codes for a fusion protein between the estradiol binding domain of the estrogen receptor (29) and the ΔN3-S218E-S222D form of MEK (17, 18a). The pCMV-β-gal expression vector was a gift from V. Sukhatme, the v-*raf* vector was a gift from U. Rapp, and the pTK-hgr selection vector was a gift from N. Hay. Plasmid DNAs were prepared by CsCl-ethidium bromide gradient centrifugation as described previously (15) or by purification through columns as instructed by the manufacturer (Qiagen, Chatsworth, Calif.). One percent Triton-based lysis buffer (TLB) contains 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM Na_2EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg of aprotinin per ml, 20 μM leupeptin, 1 mM sodium orthovanadate (pH 10.0), 1 mM EGTA (pH 8.0), 10 mM NaF, 1 mM tetrasodium PP_i, 1 mM β-glycerophosphate (pH 7.4), and 0.1 g of *p*-nitrophenyl phosphate per ml.

Cells. The H19-7 cells were generated from embryonic rat hippocampal cells (9). The cells were conditionally immortalized by stable transfection with a temperature-sensitive simian virus 40 large T antigen. H19-7 cells were grown in DMEM–10% FBS and maintained at 33°C under G418 selection throughout the experiments (9, 33). To induce differentiation, cells were placed in N2 medium and shifted to 39°C prior to treatment with differentiating agents as previously described (8).

The v-*raf*-expressing (v-raf) and ΔRaf-1:ER cells were generated by stably transfecting H19-7 cells with the vectors expressing the mutant oncogenic v-*raf* or the estradiol-regulated human Raf-1 (ΔRaf-1:ER) (29) by the calcium phosphate precipitation method as described previously (27). Cells were cotransfected with a pTK-hgr vector expressing a hygromycin resistance gene, and stable transfectants were selected for survival in the presence of 200 μg each of hygromycin and Geneticin per ml.

Raf immune complex kinase assay. Raf-1 kinase activity was analyzed by immune complex kinase assay (26). Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 10 mM Tris HCl (pH 7.4)–1% Triton X-100–1 mM EDTA–150 mM NaCl–50 mM NaF–0.1% bovine serum albumin (BSA)–20 μg of aprotinin per ml–200 μM Na_3VO_4–0.2 mM phenylmethylsulfonyl fluoride. The cell debris and nuclei were removed by centrifugation, and the supernatants were incubated with anti-c-Raf antibody (Santa Cruz) for 1.5 h at 4°C. Protein A-Sepharose was added for another 30 min. Then the mixtures were loaded onto equal volume of lysis buffer plus 10% sucrose and centrifuged for 20 min at 2,500 rpm. The pellet was washed twice with lysis buffer, twice with PAN [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 7.0), 100 mM NaCl, 20 μg of aprotinin per ml] plus 0.5% Nonidet P-40, and finally twice with PAN. The pellet was resuspended in 20 μl of kinase buffer (20 mM PIPES [pH 7.0], 10 mM $MnCl_2$, 20 μg of aprotinin per ml, 200 μM Na_3VO_4) containing 25 to 50 ng of kinase-inactive MEK-1 prepared as described below. The immunocomplex kinase reaction was initiated by adding 10 μCi of [γ-^{32}P]ATP to the mixture and incubated at 30°C for 15 to 20 min. The reaction was terminated by adding 2× sodium dodecyl sulfate (SDS)-gel sample buffer and boiled for 2 to 5 min. The samples were resolved on an

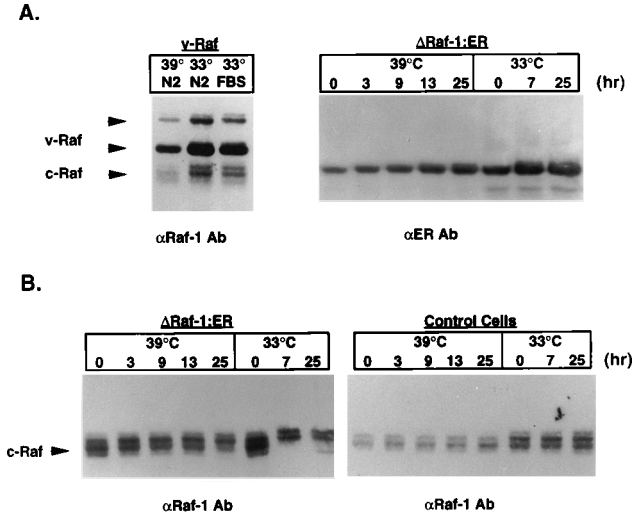

FIG. 2. Immunoblots depicting expression of v-Raf or ΔRaf-1:ER protein in v-raf or ΔRaf-1:ER cells. (A, left) v-raf cells grown at 33°C in DMEM–10% FBS were either assayed directly or switched to N2 medium and left at 33 or 39°C for 2 days. Cell lysates were prepared as described in Materials and Methods. Twenty micrograms of cellular protein was loaded in each lane and resolved by SDS-PAGE. Endogenous Raf-1 and ectopic v-Raf proteins were probed with an anti-Raf-1 antibody (C-12) against the 12 carboxy-terminal amino acids of human Raf-1. The hybridized bands were detected by ECL and exposed to X-ray film for autoradiographs. The endogenous Raf and the ectopically expressed v-Raf proteins in the transfectants are indicated. (A, right) ΔRaf-1-ER cells were grown at 33°C in DMEM–10% FBS, switched to N2 medium at either 33 or 39°C for 1 day, and then treated with 1 μM estradiol for the indicated times. Twenty micrograms of cell extract from the cells was loaded into each lane and analyzed by Western blotting with an antibody against the estrogen binding domain of estrogen receptor as a probe. (B, left) The membrane for detecting ΔRaf-1:ER expression (see panel A) was stripped and subsequently used for Western blot analysis with an anti-Raf-1 antibody. Multiple Raf-1 bands representing different levels of Raf-1 phosphorylation were detected. (B, right) Cell extracts from the control cells (H19-7 cells transfected with vector alone) were treated as for panel A (right) and then analyzed by Western blotting and probed with an anti-Raf-1 antibody. These results are representative of two or more independent experiments.

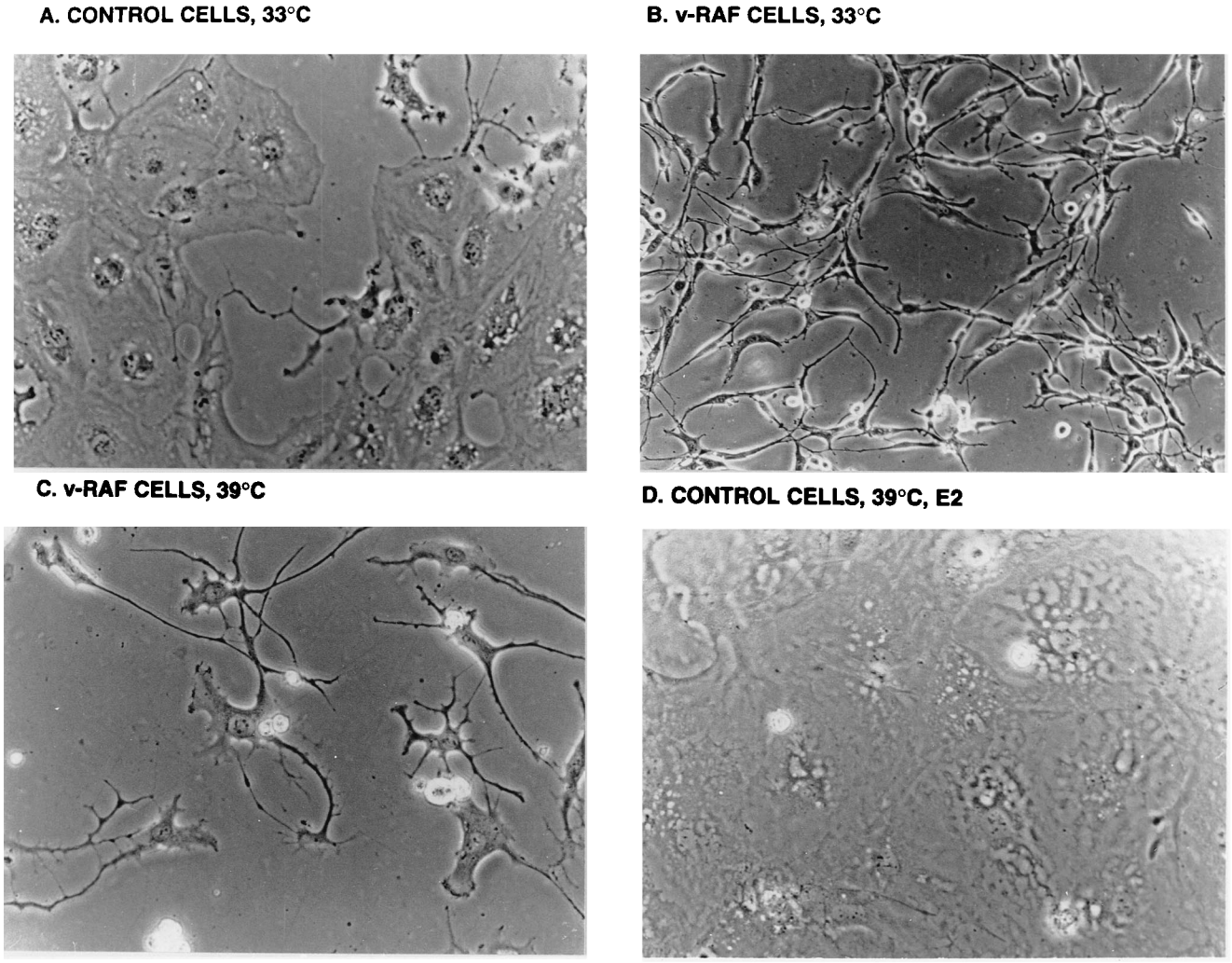

FIG. 3. Differentiation of H19-7 cells transfected with v-*raf-1* or ΔRaf-1:ER. H19-7 cells were transfected with v-*raf* or ΔRaf-1:ER plasmid DNA, and stable transfectants resistant to hygromycin were selected. Cells were grown as indicated and visualized by phase-contrast microscopy. (A) Control cells resistant to hygromycin, N2 medium, 33°C, original magnification of ×200; (B) v-raf cells in DMEM–10% FBS, 33°C, ×100; (C) v-raf-1 cells, N2 medium, 39°C, ×200; (D) control cells treated with 1 μM estradiol (E2) for 24 h, 39°C, ×200; (E) ΔRaf-1ER cells, no estradiol, 39°C, ×200; (F) ΔRaf-1:ER cells treated with 1 μM estradiol for 12 h, 39°C, ×200; (G). ΔRaf-1:ER cells treated with 1 μM estradiol for 24 h, 33°C, ×200; (H) ΔRaf-1:ER cells treated with 1 μM estradiol for 24 h, 39°C, ×200.

SDS–10% gel, and the level of MEK phosphorylation was measured by scanning the dried gels directly with an AMBIS (San Diego, Calif.) image scanner.

To assay ΔRaf-1:ER kinase activity, 200 μg of cellular lysate was incubated with 1.4 μg of rat anti-estrogen receptor antibody H222 (kindly provided by Geoffrey Greene) for 90 min at 4°C. Thirty microliters of a 1:1 slurry of protein A-Sepharose precoupled with rabbit anti-rat IgG (Sigma) was added to each tube containing the cellular lysate to precipitate the immunocomplexes. ΔRaf-1:ER kinase activity in immunocomplexes was assayed as in the same manner as described above for Raf-1 kinase activity.

Purification of inactive MEK-1 fusion protein. The His_6–MEK-1 fusion protein was purified essentially as described by Robbins et al. (25), with modifications. Briefly, BL21-DE3 cells (Novagen, Madison, Wis.) transformed with pREST-MEK1 (kindly provided by Gary Johnson) were grown in 2×YT (16 g of tryptone, per liter, 10 g of yeast extract, and 5 g of NaCl per liter) at 30°C until the optical density at 540 nm reached 0.4 to 0.5. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce the expression of histidine-tagged MEK-1 fusion protein for 4 to 5 h. Cells were harvested and lysed in a sonication buffer containing 50 mM $NaPO_4$ (pH 7.8) and 0.3 M NaCl. The lysate was incubated with nickel beads (Ni-nitrilotriacetic acid; Qiagen) and washed with the sonication buffer containing 25 mM imidazole, and then the fusion protein was eluted with stepwise increases of imidazole concentrations from 50 to 750 mM. The eluted fusion proteins were further purified by using Centricon-30 columns. The purified inactive MEK-1 protein was stored at 100 ng/μl in a storage buffer containing 20 mM Tris (pH 7.5), 1 mM dithiothreitol, (DTT), 1 mM EGTA, 10 mM benzamidine, and 10% glycerol.

Analysis of endogenous MAPK activity. (i) In-gel renaturation assay. MAPK activity was analyzed by an in-gel renaturation assay (5). Cell extracts (5 to 10 μg of total protein) were resolved in an SDS–10% polyacrylamide gel containing 0.1 mg of MBP per ml. After electrophoresis, the gel was washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–5 mM β-mercaptoethanol (buffer A) plus 20% 2-propanol to remove SDS. The gel was then reequilibrated with buffer A at room temperature for an hour, which was followed by two changes of 6 M guanidine HCl in buffer A to denature the proteins. Subsequently, the enzymes on the gel were renatured with two changes of 500 ml of buffer A containing 0.04% Tween 20 at 4°C for 16 h. The renatured gel was then incubated with buffer B containing 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM sodium vanadate, and 5 mM β-mercaptoethanol at 30°C for 30 min. The phosphorylation assay was conducted by layering 10 ml of buffer B plus 50 μM [γ-^{32}P]ATP (250 μCi) onto the renatured gel and incubating the gel at 30°C for 1 h. The reaction was terminated by immersing the gel in 5% trichloroacetic acid and 10 mM sodium pyrophosphate, and the gel was washed extensively with this solution to remove unreacted radioactivity. Autoradiograms were developed by exposing the X-ray film to the dried gels with an intensifying screen. Quantitation of the MBP phosphorylation by the MAPKs was measured by directly scanning of the dried gels with an AMBIS image analyzer.

(ii) ERK immunoprecipitation assay. One hundred micrograms of cellular lysate was incubated with 2 μl of ERK-specific antibodies and assayed as previ-

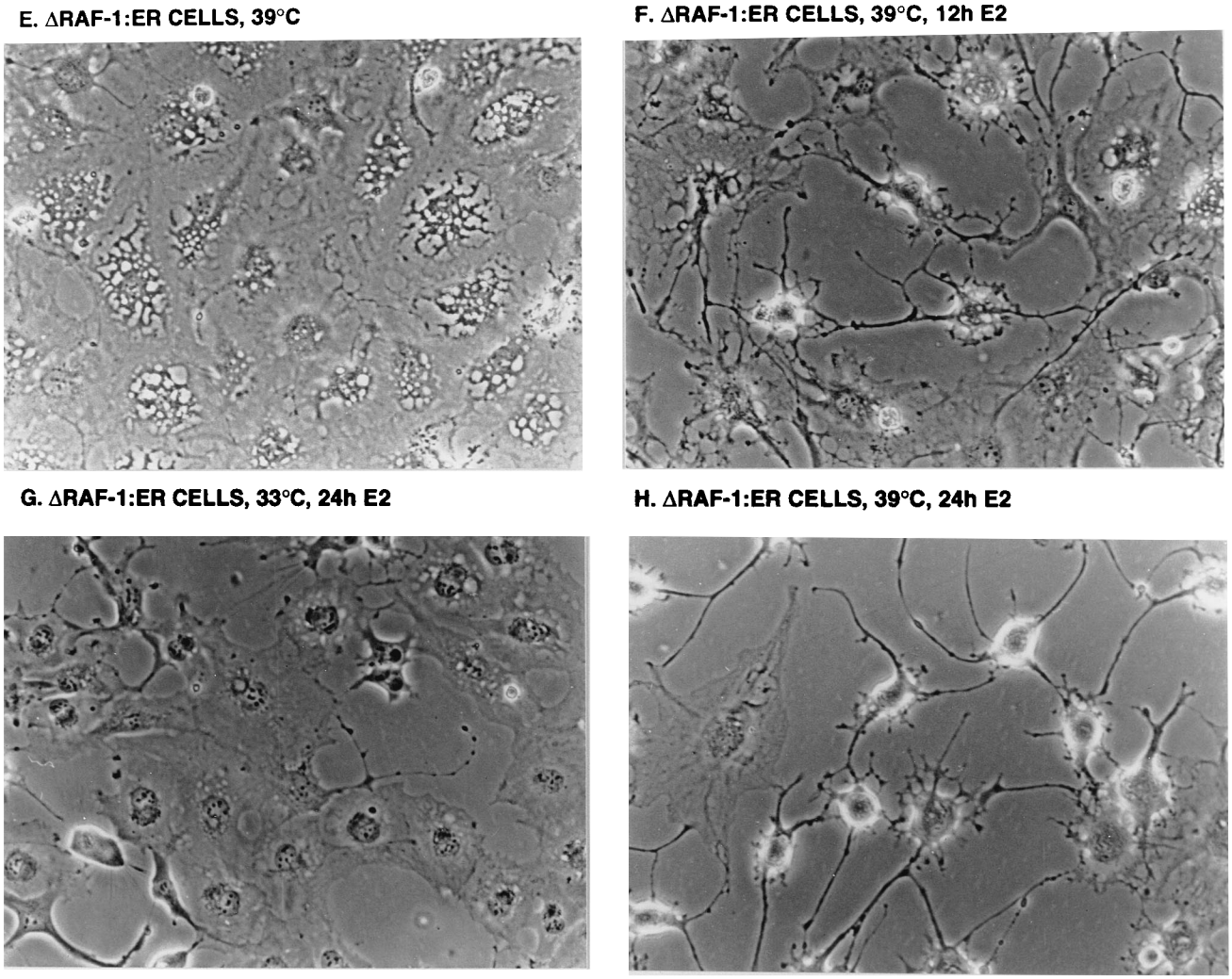

FIG. 3—*Continued.*

ously described (28) except that the reaction buffer used in this experiment contained 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 200 μM sodium vanadate, 10 mM *p*-nitrophenyl phosphate, 0.25 mg of MBP per ml, 50 μM ATP, and 5 μCi of [γ-^{32}P]ATP.

Assay of neurofilament expression. High- and middle-molecular-weight neurofilament proteins were detected by immunocytochemistry as previously described (8).

Assay of endogenous MEK activity. Cells were treated with estradiol for different lengths of time at either 33 or 39°C, and cell extracts were prepared with 1% TLB as described in the assay for MAPK. Briefly, 30 μg of protein was incubated with 2 μg of inactive ERK1 protein in a reaction mixture containing 40 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, and 10 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The reaction mixture was separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and exposed to X-ray film for autoradiography. Membranes were subsequently probed with an antiphosphotyrosine antibody and detected by enhanced chemiluminescence (ECL).

Assay of MEK-2E activity. ΔRaf-1:ER cells were plated at 3×10^5/100-mm-diameter dish 1 day before transfection. Plasmid EE-MEK-2E-CMV (38) and HA-tagged ERK2 (HA-ERK2) (3), 15 and 5 μg, respectively, were transfected into ΔRaf-1:ER cells by the calcium phosphate precipitation method. The empty vector, EE-CMV, was used as a control plasmid. Eighteen hours after transfection and glycerol shock, cells were switched to N2 medium and shifted to 39°C; 24 h after the temperature shift, cells were treated with either EGF (10 nM) or bFGF (10 ng/ml) for 5 min or with estradiol (1 μM) for 30 min and lysed in 500 μl of 1% TLB. The HA-ERK2 proteins in the lysates were immunoprecipitated with a monoclonal antibody, 12CA5, against the HA epitope tag and used for assaying phosphorylation of MBP in vitro (see below).

HA-ERK2 kinase assay. ERK2 kinase activity was assessed by immunoprecipitation of the epitope tag followed by an in vitro phosphorylation assay (20). Transfected cells were stimulated and lysed with 0.5 ml of ice-cold 1% TLB. Insoluble materials were removed by centrifugation at 14,000 rpm for 10 min at 4°C. The cell lysates were then incubated with 25 μl of protein A-Sepharose precoupled with antibody 12CA5 specific to the HA epitope (BabCo) for 3 to 16 h at 4°C. The immune complexes were washed twice with lysis buffer and once with kinase reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 200 μM sodium orthovanadate, and 10 mM *p*-nitrophenyl phosphate. The MBP phosphorylation reaction mixture was separated by SDS-PAGE on a 12% polyacrylamide gel and then transferred by a Hoefer Semi-Phor transfer apparatus to a nitrocellulose membrane, using a buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol. To quantitate the phosphorylation of MBP, the membrane was directly scanned by an AMBIS image system. The nitrocellulose was blocked with a solution of 5% BSA in 50 mM Tris (pH 7.5)–150 mM NaCl–0.2% Tween 20. Blots were probed with a 1:500 dilution of antibody 12CA5 for detecting the expression of HA-tagged protein. Western blots (immunoblots) were visualized by using ECL reagents (Amersham, Arlington Heights, Ill.).

Transient transfection of H19-7 and ΔRaf-1:ER cells. About 10^5 cells (H19-7 for ΔRaf-1:ER expression and ΔRaf-1:ER cells for MEK-2E or ΔMEK1:ER expression) were seeded in each well in a six-well plate. The next day, cells in each well were cotransfected with 2.5 μg of EE-MEK2E-CMV or pLNCΔMEK1:ER and 2.5 μg of pCMV-β-gal expression vector by the calcium phosphate precipitation method (27). At 12 to 18 h after transfection, cells were switched to N2 medium and shifted to 39°C. At the same time, estradiol, bFGF, or vehicle was added to the N2 medium to differentiate the cells. About 24 to 36 h after the temperature shift and the treatment, cells were fixed and stained for β-galactosidase activity (16). Cells expressing β-galactosidase stained blue and were categorized for differentiation state according to their morphologies and

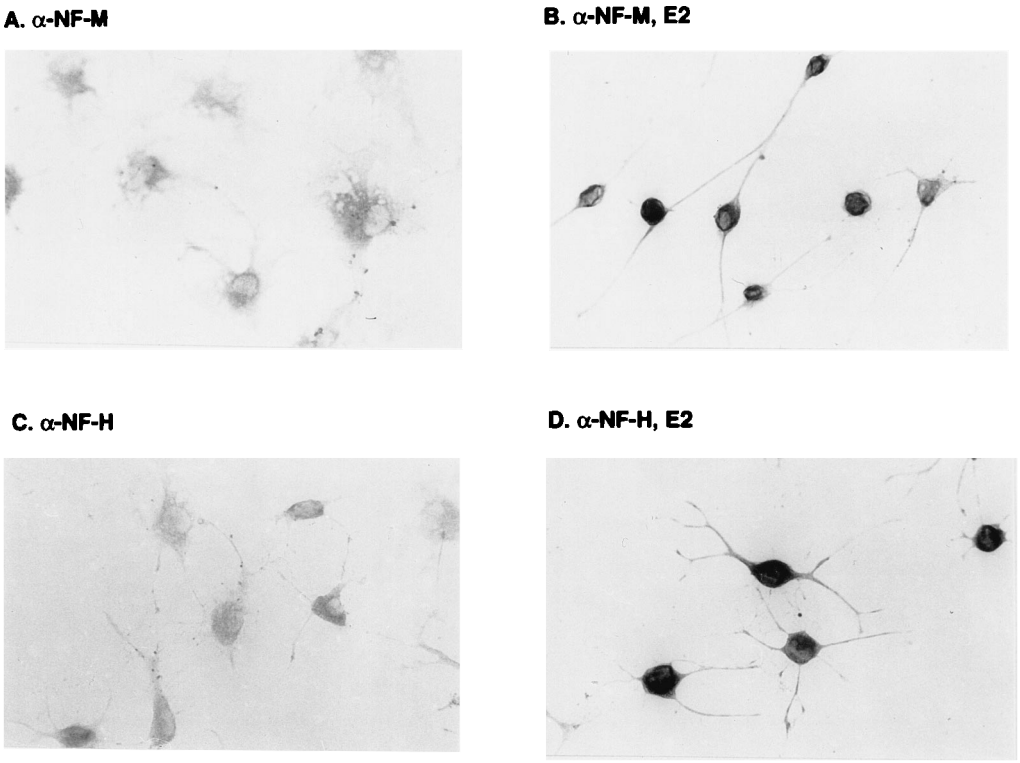

FIG. 4. Expression of high- and medium-molecular-weight neurofilament proteins in ΔRaf-1:ER cells. ΔRaf-1:ER cells were grown on polylysine-coated coverslips in DMEM–10% FBS at 33°C, switched to N2 medium at 39°C for 12 h, and then treated or not treated with 10 nM estradiol (E2) for 36 h. Cells were fixed and stained with an antibody against high-molecular-weight neurofilaments (α-NF-H) or an antibody against medium molecular weight neurofilaments (α-NF-M) as described in Materials and Methods. (A) Untreated ΔRaf-1:ER cells stained with α-NF-M; (B) ΔRaf-1:ER cells treated with estradiol and stained with α-NF-M; (C) untreated ΔRaf-1:ER cells stained with α-NF-H; (D) ΔRaf-ER cells treated with estradiol and stained with α-NF-H. The pictures were taken at a magnification of ×200.

the lengths of their processes. Differentiated cells were defined as those cells that extended neurites longer than the length of the cell body. For transfections with the pLNCΔMEK1:ER construct, a total of 90 cells were analyzed in two independent experiments. Pictures were taken with a Leitz DMIRB microscope and a Nikon 35-mm camera.

Microinjection of H19-7 and Raf-1:ER cells. About 4×10^4 cells were seeded on polylysine-coated coverslips placed in 12-well dishes. After the cells grew to desired densities, the cells were microinjected with plasmid DNA and rat IgG in injection buffer (50 mM HEPES [pH 7.4], 40 mM NaCl), using Eppendorf equipment (micromanipulator 5171, microinjector 5246, and Femtotips) and a Leitz DMIRB microscope.

Indirect immunofluorescence labeling. At 24 to 48 h after microinjection, the cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and then treated with permeabilizing solution (0.2% [wt/vol] Triton X-100 in 2% formaldehyde–PBS) for 5 min. Fixed cells were blocked with 15% goat serum in PBS for 15 min. Incubations with antibodies against estrogen receptor (10 μg/ml) and β-galactosidase (1:400 dilution; 5 Prime → 3 Prime, Inc.) were carried out at 4°C overnight or at room temperature for 1 h. Fluorescein isothiocyanate- and Texas red-conjugated goat anti-rabbit IgG or anti-rat IgG (Cappel, Durham, N.C.) was added, and the mixture was incubated for 1 h at room temperature. Between each of the foregoing steps, the sample was extensively washed with PBS. Coverslips with stained cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.) and examined with a Leitz DMIRB microscope. Images were captured with a charge-coupled device video camera using the public domain NIH image program (National Institutes of Health) and printed with a Sony color printer.

RESULTS

FGF induces prolonged ERK activation in rat hippocampal H19-7 neuronal cells. Rat hippocampal neuronal H19-7 cells were shifted to 39°C to inactivate the simian virus 40 T antigen, transferred to serum-free N2 medium for 2 days, and then treated either with 10 ng of FGF per ml, which will differentiate the cells within 2 days, or 10 nM EGF, which will not differentiate the cells (9, 33). To determine the potential involvement of MAPKs (ERK1 and -2) in FGF-induced differentiation, cell extracts were obtained from 0 to 5 h following treatment and analyzed for ERK activation by a gel shift assay that measures the state of enzyme phosphorylation (Fig. 1). These results show that ERKs are rapidly activated by both FGF or EGF treatment, but that the activation is prolonged (albeit at a lower level) for up to 5 h in the FGF-treated sample only. These results are consistent with previous results obtained with PC12 cells that have indicated a role for prolonged ERK activation in promoting differentiation of neuronal cells.

Activated Raf-1 induces neuronal differentiation of H19-7 cells. A major pathway by which ERK is activated by tyrosine kinase growth factor receptors involves sequential activation of Ras, Raf-1, and MEK (reviewed in reference 18). To determine whether activated Raf-1 is sufficient to induce neuronal differentiation of H19-7 cells, cells were stably transfected with v-*raf* at 33°C. Immunoblot analysis of the clones with anti-Raf-1 antibody showed expression of v-*raf* both at 33°C in serum and at 33 and 39°C in N2 medium (Fig. 2). Two forms of the v-*raf* product, one containing and one lacking the Gag fusion protein, are expressed as previously described (24). Many of the clones display neurite extension even at 33°C, but shifting the cells to 39°C to inactivate T antigen resulted in more extensive neurite formation (Fig. 3A to C). These results indicate that activated Raf-1 can induce neuronal differentiation of H19-7 cells.

To analyze the initial signals generated by Raf-1, H19-7 cells were transfected with a vector expressing ΔRaf-1:ER (29). Upon exposure to estradiol, ΔRaf-1:ER is activated within

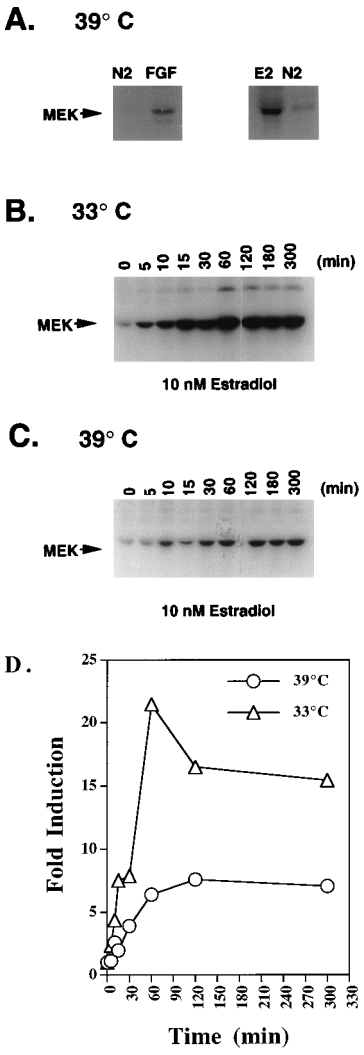

FIG. 5. Induction of Raf-1 kinase activity by bFGF in H19-7 cells and by estradiol in ΔRaf-1:ER cells. Endogenous Raf-1 proteins and ectopically expressed ΔRaf-1:ER fusion proteins were immunoprecipitated with an anti-Raf-1 antibody and with H222, an antibody against the ligand binding domain of estrogen receptors, respectively. Raf-1 activity was assayed with inactive MEK as a substrate as described in Materials and Methods. The ^{32}P-labeled MEK-1 bands are indicated. (A) Autoradiographs illustrating induction of Raf activity by FGF or estradiol at the indicated temperatures. Left, untreated H19-7 cells in N2 medium (N2) or cells treated with 10 ng of bFGF per ml for 5 min (FGF); right, untreated ΔRaf-1:ER cells in N2 medium (N2) or cells treated with 1 μM estradiol for 10 min (E2). (B) Autoradiograph illustrating time course of induction of Raf activity in ΔRaf-1:ER cells at the permissive temperature. Cells were grown in N2 at 33°C for 1 day and then treated with 10 nM estradiol for the times indicated. (C) Autoradiograph illustrating time course of induction of Raf activity in ΔRaf-1:ER cells at the nonpermissive temperature. Cells were grown in N2 at 39°C for 1 day and then treated with 10 nM estradiol for the times indicated. (D) Graph depicting the time course of Raf activation in ΔRaf-1:ER cells. The dried gels used to obtain the autoradiographs for panels B and C were scanned directly with an AMBIS image system. The ΔRAF-1:ER kinase activity at each time point following treatment with 10 nM estradiol at either 33 or 39°C is shown as fold induction compared with the initial activity (no estradiol). The results are representative of two independent experiments.

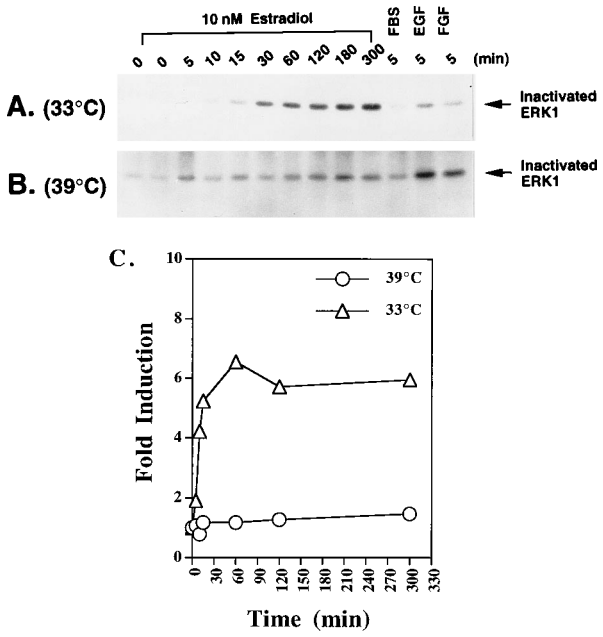

FIG. 6. Time course of stimulation of MEK activity by estradiol in ΔRaf-1:ER cells. Cells were grown as described in the legend to Fig. 2. Thirty micrograms of cell extract was assayed for MEK activity, using inactivated ERK1 as a substrate as described in Materials and Methods. The reaction mixture was resolved by SDS-PAGE, blotted onto a nitrocellulose membrane, and exposed to X-ray film for autoradiography. The ^{32}P-labeled ERK1 bands are indicated. (A) MEK activation by 10 nM estradiol at 33°C. Exposure time for the autoradiograph was 1 h at −80°C. (B) MEK activation by 10 nM estradiol at 39°C. Exposure time for the autoradiograph was 15 h at −80°C. (C) Graph depicting time course of MEK activation in ΔRaf-1:ER cells. The membranes used for panels A and B were directly scanned with an AMBIS image system. The MEK activity at each time point following treatment with 10 nM estradiol at either 33 or 39°C is shown as fold induction compared with the initial activity (no estradiol). The results are representative of two independent experiments.

minutes, enabling one to monitor early signaling events (29) (see Fig. 5). Analysis of stably transfected cells with an antibody to the estrogen receptor showed expression of ΔRaf-1:ER protein at both 33 and 39°C (Fig. 2A, right). After addition of 1 μM estradiol to the ΔRaf-1:ER cells, the amount of ΔRaf-1:ER protein increased with time. As has been reported previously (29), rapid changes in the phosphorylation state of the endogenous Raf-1 protein yielding a multiplicity of bands can be detected in immunoblots with anti-Raf-1 antibody (which does not detect the ΔRaf-1:ER fusion protein) following activation of ΔRaf-1:ER, particularly at 33°C (Fig. 2B). Activation of ΔRaf-1:ER with 1 μM estradiol for 12 to 24 h caused the cells to differentiate at 39°C, as judged by morphology and neurite extension (Fig. 3F and H), whereas no significant differentiation occurred in the absence of estradiol under these conditions (Fig. 3E). Concentrations of estradiol as low as 10 nM also promoted cell differentiation (Fig. 4), but the rate of process formation (36 to 48 h) was slower, comparable to that observed when FGF was used to differentiate the cells. Like the v-*raf*-expressing cells, the ΔRaf-1:ER-expressing cells at 33°C (the temperature at which T antigen is active) are also capable of morphological differentiation, but only after prolonged activation of ΔRaf-1:ER by estradiol for at least 3 days (Fig. 3G; data not shown). Estradiol treatment of mock-transfected H19-7 cells had no effect on cell differentiation (Fig. 3D). Differentiation of the ΔRaf-1:ER cells by estradiol was confirmed by immunostaining for neurofilament expression (Fig. 4). When cells were switched to 39°C for 12 h and then 10 nM estradiol was added for an additional 36 h, significant induction of both high- and middle-molecular-weight neurofilament expression was observed.

To determine whether the differentiation by FGF or ΔRaf-1:ER was due to activation of Raf-1, the activity of immunoprecipitated Raf was measured by using MEK as a substrate.

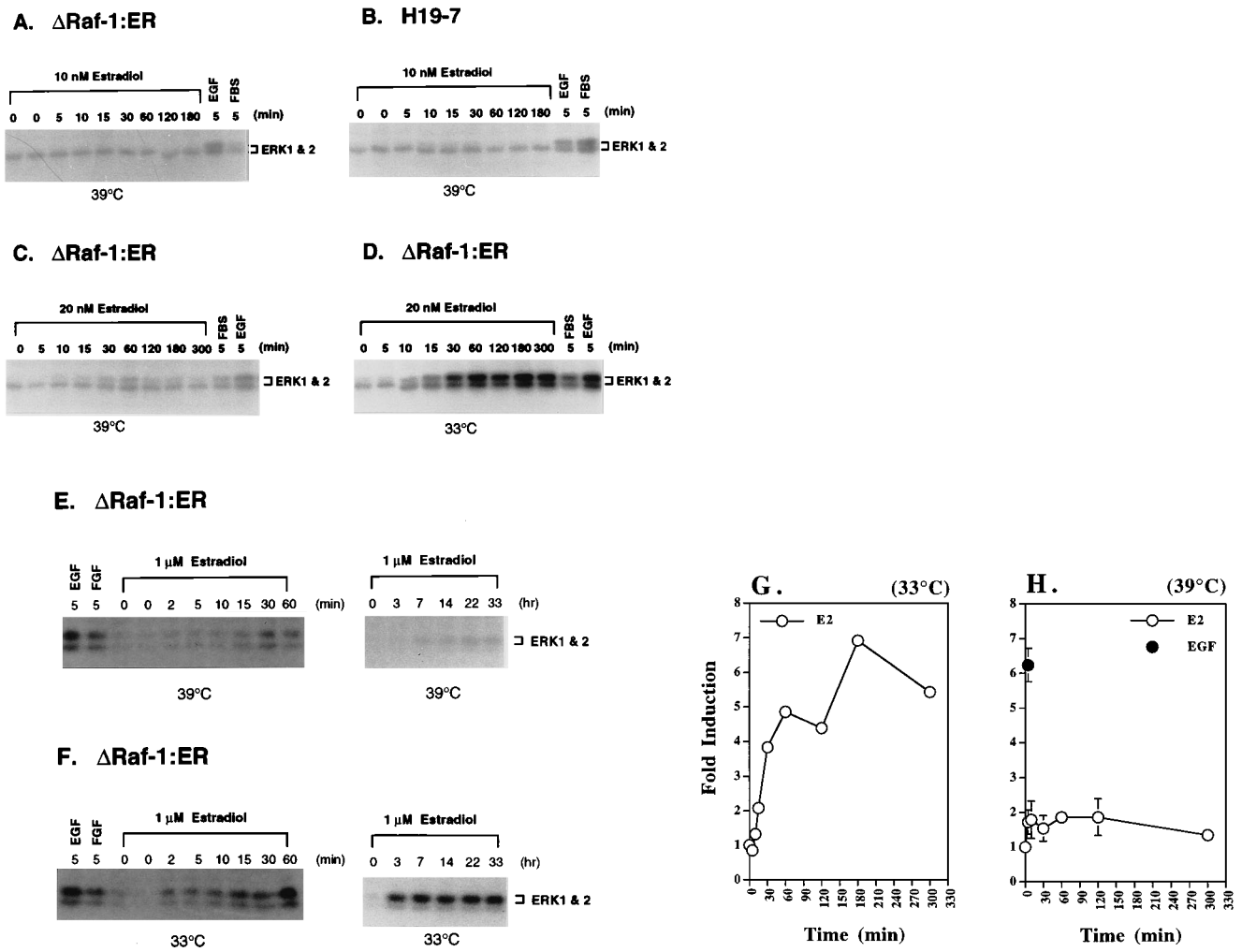

FIG. 7. Time course of stimulation of ERK activity by estradiol in ΔRaf-1:ER cells. Cells were grown as described in the legend to Fig. 2. Ten micrograms of cell extract was loaded in each lane, and ERK activity was assayed by the in-gel kinase assay with MBP as a substrate as described in Materials and Methods. The gels were dried and then exposed to X-ray film for autoradiography. (A) ΔRaf-1:ER treated with 10 nM estradiol, 39°C; (B) control cells (H19-7) treated with 10 nM estradiol, 39°C; (C) ΔRaf-1:ER cells treated with 20 nM estradiol, 39°C; (D) ΔRaf-1:ER cells treated with 20 nM estradiol, 33°C; (E) ΔRaf-1:ER cells treated with 1 μM estradiol, 39°C; (F) ΔRaf-1:ER cells treated with 1 μM estradiol, 33°C. The phosphorylated bands corresponding to ERK1 and -2 are indicated. (G and H) Graphs depicting time course of ERK activation in ΔRaf-1:ER cells induced by 20 nM estradiol at 33°C (G) or in ΔRaf-1:ER cells induced by 10 nM estradiol (H). Samples in panel G were assayed by in-gel renaturation, and samples in panel H were assayed with immunoprecipitated ERK as described in Materials and Methods. The radioactivities of the ^{32}P-labeled ERK bands were detected and analyzed with an AMBIS image system. The ERK activity at each time point following treatment with estradiol at either 33 or 39°C is shown as fold induction compared with the initial activity (no estradiol). The results are representative of two independent experiments.

As shown in Fig. 5A, at 39°C, endogenous Raf-1 activity was detectable in H19-7 cells treated with FGF, and ΔRaf-1:ER activity was detected in ΔRaf-1:ER cells treated with estradiol. Addition of 10 nM estradiol to cells that had been in N2 medium at 39°C for 2 days resulted in >6-fold ΔRaf-1:ER activation by 60 min (Fig. 5C and D). Similar induction of ΔRaf-1:ER activity by estradiol was observed at 33°C, but the level of ΔRaf-1:ER activity at this temperature was approximately two- to threefold higher, reflecting in part the higher level of protein at that temperature (Fig. 5B and D). The ΔRaf-1:ER activity was specifically due to the expression of the ΔRaf-1:ER construct, as shown by the lack of activity in anti-estrogen receptor immunoprecipitates from cells treated with either FGF or EGF or with serum (data not shown). Furthermore, no significant endogenous Raf-1 activity was detected in response to 10 nM estradiol in the parent H19-7 cells (data not shown).

Temperature-dependent activation of MEK and ERKs by Raf. The extent of MEK activation in response to ΔRaf-1:ER stimulation was dependent on the temperature (Fig. 6). At 33°C, the temperature at which T antigen is active, stimulation of ΔRaf-1:ER with 10 nM estradiol resulted in the constitutive activation of MEK, as assayed by phosphorylation of kinase-inactive ERK1 in crude cell extracts. Similar results were obtained when MEK activity was determined by Western blot analysis of tyrosine-phosphorylated kinase-inactive ERK1 or when MEK was immunoprecipitated and assayed directly for activity (data not shown). The level of MEK activation (increase of >5-fold) at 33°C in response to estradiol-stimulated ΔRaf-1:ER was comparable to that observed after treatment of the cells for 5 min with either EGF or serum. On the other hand, when cells were shifted to 39°C to inactivate T antigen, only low MEK activity (<2.0-fold induction) was detectable in response to 10 nM estradiol-induced ΔRaf-1:ER. Thus, MEK activity was induced in response to estradiol treatment at 39°C,

but it was significantly lower than the comparable ΔRaf-1:ER activity.

MAPK (ERK1 and -2) activation in the ΔRaf-1:ER cells in response to estradiol was monitored at both 33 and 39°C, using an in-gel renaturation assay (Fig. 7). After induction of ΔRaf-1:ER at 33°C with 1 μM estradiol, ERK1 and -2 were constitutively activated from 2 min after estradiol addition until at least 33 h following treatment. The levels of ERK activation at 33°C were as high as or higher than after treatment with FGF for 5 min (Fig. 7F). At 39°C, however, ERK activation in response to 1 μM estradiol induction of ΔRaf-1:ER was less robust, peaked around 30 min, and was barely detectable by 3 h after treatment (Fig. 7E). Although estradiol treatment of the parent H19-7 cells stimulated some activation of ERK as well (Fig. 7B), this was insufficient to induce differentiation.

The temperature-dependent difference in ERK activation was even more apparent at low estradiol concentrations. At 33°C, ΔRaf-1:ER cells treated with 20 nM estradiol showed detectable ERK activity by 10 min after treatment, which reached a peak (>5-fold) by 1 h and remained robust even after 5 h of estradiol treatment (Fig. 7D and G). In contrast, induction at 39°C of ΔRaf-1:ER with 10 to 20 nM estradiol yielded a low increase in ERK activity over background, comparable to that observed in the parent H19-7 cells in response to 10 nM estradiol (Fig. 7A to C). To gain a more accurate estimation of the level of ERK stimulation, ERK activity in immunoprecipitates was also assayed directly. Induction at 39°C of ΔRaf-1:ER with 10 nM estradiol yielded a small increase in ERK activity over background (<2-fold) (Fig. 7H). These results indicate that ERK-1 and -2 are constitutively activated at 33°C, the temperature at which T antigen is active, and are also stimulated but to a significantly lower extent at 39°C.

Transiently transfected MEK will not induce neuronal differentiation. The previous results suggest that prolonged MAPK activation may not be sufficient for neuronal differentiation of H19-7 cells within 36 h of estradiol treatment at 33°C. To determine whether constitutive MAPK activity is sufficient to induce differentiation of cells at 39°C in the absence of an immortalizing gene, constitutively activated MEK was introduced into cells by either transient transfection or microinjection. Initially, the effect of transfected MEK on ERK activation was tested. ΔRaf-1:ER cells were transfected transiently at 33°C with HA-ERK2 and either a control plasmid (CMV-EE) or constitutively activated MEK-2E expression plasmid that encodes a protein with two glutamic acid residues substituted at the sites of the activating serine residues. Cells were either left at 33°C or transferred to 39°C in N2 medium. Before harvesting, cells were treated with estradiol for 30 min or with growth factor (EGF or FGF) for 5 min. At 36 h following transfection with MEK-2E and HA-ERK, the HA-ERK2 was immunoprecipitated and assayed for kinase activity, using MBP as a substrate. As shown in Fig. 8, expressed MEK-2E was effective at generating constitutively active MAPK at both 33 and 39°C, and the activity was comparable to that induced by growth factor treatment of the cells (Fig. 8A and B). Transfection with ΔMEK1:ER, a plasmid expressing activated MEK fused to the estradiol binding domain of the estrogen receptor, stimulated HA-ERK2 to the same extent as MEK-2E at 39°C (data not shown). Higher HA-ERK2 activity in MEK-2E-expressing cells was not due to differences in HA-ERK2 protein expression (Fig. 8A, lower panel).

To determine the phenotype of the cells expressing MEK-2E, cells were cotransfected with MEK-2E and pCMV-β-gal as a reporter gene. At 12 h following transfection, cells were

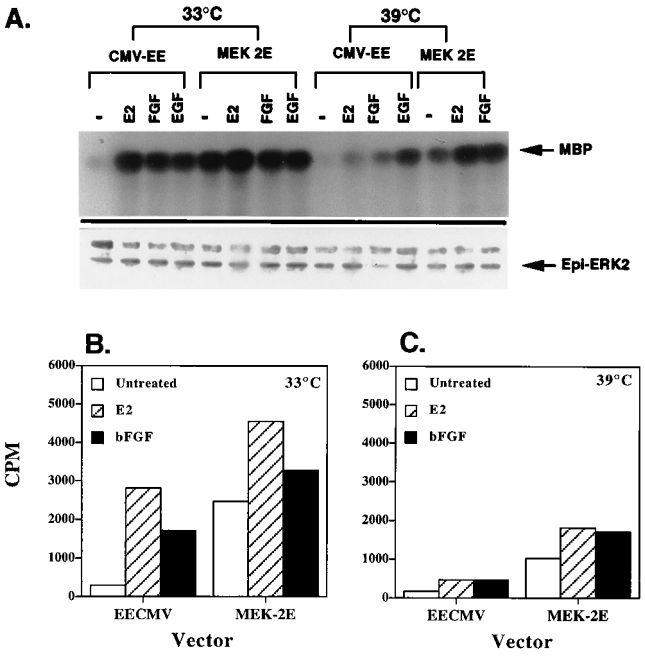

FIG. 8. Expression of constitutively active MEK-2E by transient transfection. (A) Fifteen micrograms of MEK-2E mammalian expression vector or control vector (CMV-EE) and 5 μg of HA-ERK2 expression vector were transfected into ΔRaf-1:ER cells as described in Materials and Methods. Before harvesting, cells were left untreated (−) or treated with 1 μM estradiol for 30 min (E2) or with growth factors for 5 min (EGF or bFGF) at the indicated temperatures. HA-ERK activity was assayed with MBP as a substrate as described in Materials and Methods. The reaction mixtures were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane, and exposed to a X-ray film for autoradiography. The ^{32}P-labeled MBP bands are indicated with an arrow. The same membrane was subsequently used for the detection of immunoprecipitated HA-ERK2 protein with 12CA5, a peroxidase-conjugated secondary antibody, and ECL as described in Materials and Methods. HA-ERK2 (Epi-ERK2) protein is indicated with an arrow. The bands above the HA-ERK2 proteins are mouse immunoglobulin heavy chains. (B and C) Graphs of ERK activity at the indicated temperatures. The ^{32}P-labeled MBP bands in panel A were quantitated by directly scanning the membrane with an AMBIS image system. The results are representative of two independent experiments.

shifted to 39°C in N2 medium and monitored during the next 24 to 48 h for differentiation as defined by morphological changes and neurite formation. Initially, the ΔRaf-1:ER vector was transfected into H19-7 cells as a control for the differentiation efficiency. In the presence of 1 μM estradiol, 47% of the β-galactosidase-expressing cells were differentiated (Fig. 9C and D; Table 1). In contrast, in the absence of estradiol, 90% of the β-galactosidase-expressing cells counted were undifferentiated (Fig. 9A and B; Table 1). Furthermore, 95% of the cells that did not express β-galactosidase activity were undifferentiated. These results confirm that transient expression and activation of ΔRaf-1:ER led to differentiation of H19-7 cells.

When the MEK-2E vector was cotransfected with β-galactosidase into ΔRaf-1:ER cells, 87% of the β-galactosidase-expressing cells remained undifferentiated in the absence of estradiol (Fig. 9E and F; Table 1). After induction of ΔRaf-1:ER with 1 μM estradiol, 83% of the β-galactosidase-expressing cells were differentiated. Similar results were obtained after bFGF treatment (data not shown), indicating that the transfected cells are capable of undergoing differentiation. To confirm that the majority of cells expressing β-galactosidase also expressed the transfected MEK, similar experiments were conducted with the ΔMEK1:ER construct instead of MEK-2E.

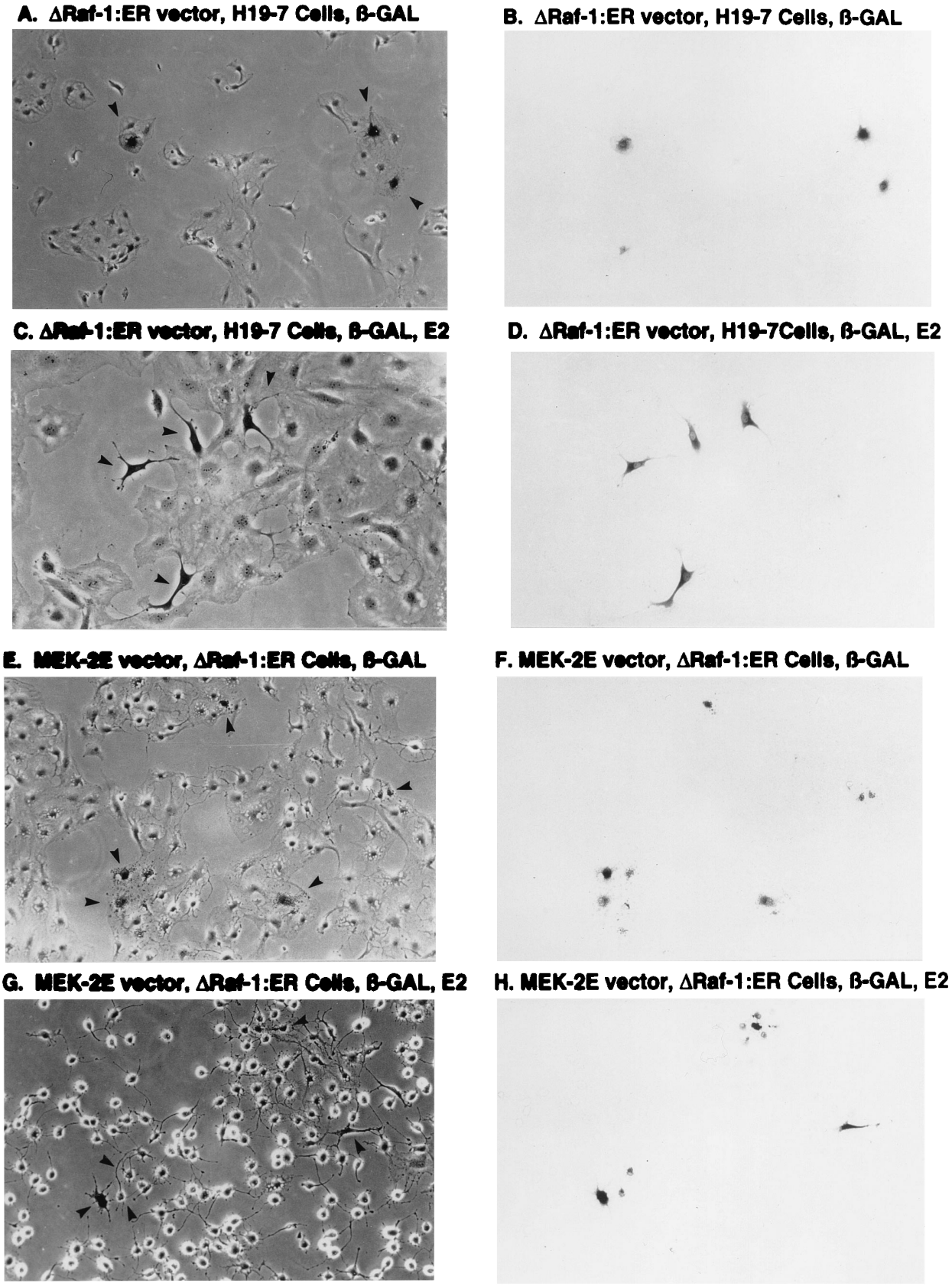

FIG. 9. Characterization of 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal)-stained cells cotransfected with MEK-2E and pCMV-β-gal vectors. H19-7 (A to D) or ΔRaf-1:ER (E to H) cells were transfected with a ΔRaf-1:ER (A to D) or MEK-2E (E to H) expression vector along with pCMV-β-gal (1:1). After being shifted to 39°C in N2 medium, the cells were either untreated (A, B, E, and F) or treated with 1 μM estradiol (E2) (C, D, G, and H). Cells were assayed for β-galactosidase expression and staining with X-Gal as described in Materials and Methods. Cells were visualized by either phase-contrast microscopy (A, C, E, and G) or light microscopy (B, D, F, and H) at a magnification of ×200. In phase-contrast micrographs (A, C, E, and G), some of β-galactosidase-expressing cells are indicated by arrowheads.

TABLE 1. Effect of MEK-2E or ΔRaf-1:ER on the differentiation of hippocampal neuronal cells[a]

Cell type/vector	% with indicated morphology				No. of β-galactosidase-expressing cells counted
	β-Galactosidase-expressing cells			Whole cell population[b]	
	Differentiated	Undifferentiated	Intermediate		
H19-7/ΔRaf-1:ER					
−Estradiol	5	90	6	>95 undiff	143
+Estradiol	47	42	12	>90 undiff	163
ΔRaf-1:ER/MEK-2E					
−Estradiol	5	87	9	>90 undiff	239
+Estradiol	83	9	7	>90 diff	174

[a] The results were obtained from two independent transfections for ΔRaf-1:ER and three independent transfections for MEK-2E.
[b] Undiff, undifferentiated; diff, differentiated.

Results from two independent experiments indicated that 74% ± 6% of the cells coexpressed ΔMEK1:ER and β-galactosidase, as determined by immunostaining with antibodies to the estrogen receptor and β-galactosidase, respectively. This finding suggests that even in the absence of functional large T antigen, constitutive ERK activation for 36 h is not sufficient for neuronal differentiation of H19-7 cells.

Microinjected MEK will not induce neuronal differentiation. To confirm these results, plasmids encoding activated MEK were also introduced into cells by microinjection, and the effect on neuronal differentiation was determined. The efficiency of expression of microinjected plasmids was initially determined by coinjecting plasmid pCMV-β-gal along with rat IgG protein into the nuclei of ΔRaf-1:ER cells. The results of two independent experiments show 89% ± 1% of the injected cells, monitored by immunostaining with antibody against rat IgG, also express β-galactosidase (Fig. 10). When plasmids ΔMEK1:ER and pCMV-β-gal were coinjected, a similar colocalization of the expressed proteins was observed by immunostaining (data not shown).

To demonstrate that the microinjected cells expressing activated MEK had elevated levels of ERK activity, ΔRaf-1:ER cells microinjected with MEK-2E were cultured for 46 h, shifted to 39°C in N2 defined medium for 2 h, and then stained with an antibody directed against the activated, phosphorylated form of ERK. In contrast to uninjected cells, in which no staining was detected (Fig. 11C and D), both cells microinjected with MEK-2E (Fig. 11A and B) and a population of uninjected cells that had been stimulated with EGF for 5 min (Fig. 11E and F) reacted with the antibody. These results indicate that microinjection of MEK-2E results in ERK activation.

Having established the efficiency and efficacy of MEK expression, we determined whether microinjected MEK induced neuronal differentiation. ΔRaf-1:ER cells were microinjected with rat IgG and the MEK-2E plasmid. After 24 h at 39°C in N2 medium, cells were immunostained with an antibody against rat IgG and evaluated for differentiation. While a control culture of uninjected ΔRaf-1:ER cells treated with 1 μM estradiol fully differentiated under these conditions (Fig. 12C), no differentiation of cells injected with MEK-2E was observed (Fig. 12A and B). Similar results were obtained when H19-7 cells were injected with a plasmid encoding ΔMEK1-ER, which transforms 3T3 cells in an estradiol-dependent manner (18b), and immunostained with an anti-estrogen receptor antibody (Fig. 13). When H19-7 cells were injected with MEK-2E, only 1 of 29 injected cells was differentiated after 3 days at 39°C in N2 medium (data not shown). These results indicate that constitutive activation of MEK and MAPK is not sufficient to induce differentiation of the H19-7 hippocampal neuronal cells at 39°C.

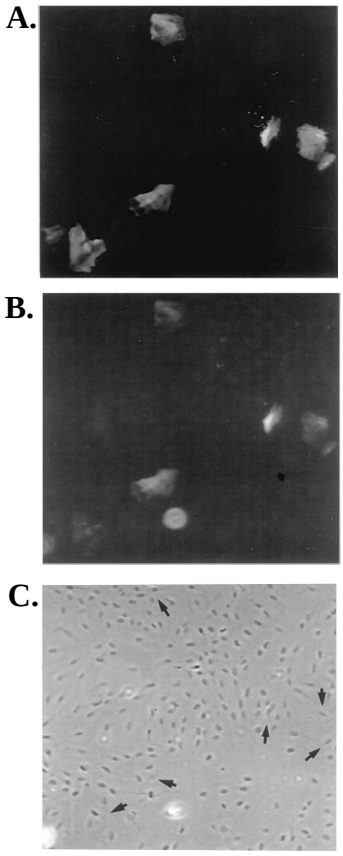

FIG. 10. Colocalization of expressed β-galactosidase and rat IgG marker injected into H19-7 cells. pCMV-β-gal DNA (0.25 μg/μl) and rat IgG (2 mg/ml) were microinjected into H19-7 cells in DMEM–10% FBS as described in Materials and Methods. Six hours after the injection, cells were switched to N2 medium at 39°C for 24 h before fixation and analysis of cells. (A) Expression of β-galactosidase as detected by rabbit anti-β-galactosidase antibody (1:400) and goat anti-rabbit fluorescein isothiocyanate-conjugated antibody (1:400); (B) rat IgG as detected by Texas-red conjugated goat anti-rat IgG (1:80); (C) phase-contrast micrograph of the same cells. Magnification, ×160. A total of 53 cells were analyzed in two independent experiments.

DISCUSSION

The studies presented here demonstrate that expression of ΔRaf-1:ER or v-*raf* can differentiate the rat hippocampal cell line H19-7. ΔRaf-1, MEK, and ERK activities following estradiol treatment remained elevated at 33°C, the temperature at which T antigen is active. Despite prolonged MEK and ERK activation at 33°C, differentiation occurred within 36 h only at 39°C. While ΔRaf-1 was highly activated in response to 10 nM estradiol at both temperatures, the activation of MEK, ERK1, and ERK2 was significantly lower when cells were shifted to 39°C to inactivate T antigen. Furthermore, expression of constitutively activated MEK, which led to constitutive activation of ERKs, was insufficient to induce differentiation at 39°C. These results suggest that prolonged activation of MEK, ERK1, and ERK2, in the presence or absence of functional T antigen, is not sufficient for differentiation of H19-7 neuronal

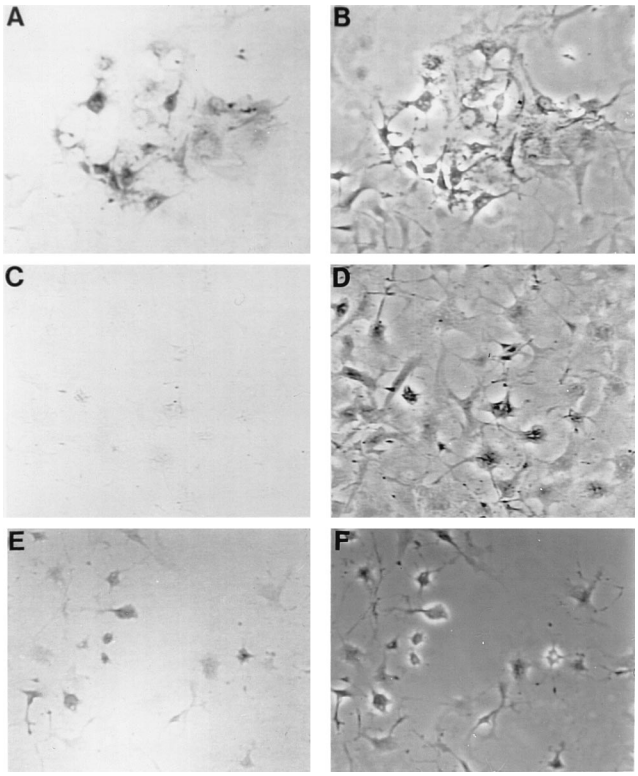

FIG. 11. Activation of ERKs in cells microinjected with activated MEK. ΔRaf-1:ER cells in DMEM–10% FBS were microinjected with the MEK-2E plasmid (1 μg/μl). The next day, cells were switched to N2 medium and incubated sequentially at 33°C for 1 day and 39°C for 2 h before fixation and analysis of cells. Phosphorylated MAPK was detected with a phosphorylated MAPK-specific antibody, peroxidase-conjugated anti-rabbit IgG, and diaminobenzidine as described in Materials and Methods. (A and B) Detection of phosphorylated MAPK in the injected cells, light and phase-contrast micrographs, respectively. (C and D) No detectable staining of phosphorylated MAPK in the uninjected cells, light and phase-contrast micrographs, respectively. (E and F) Detection of phosphorylated MAPK in uninjected ΔRaf-1:ER cells treated with 10 nM EGF for 5 min, light and phase-contrast micrographs, respectively. Magnification, ×200.

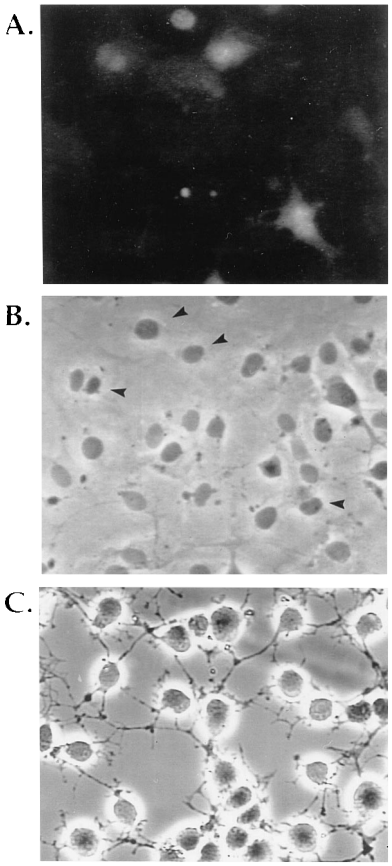

FIG. 12. Microinjection of activated MEK-2E did not differentiate ΔRaf-1:ER cells. ΔRaf-1:ER cells in DMEM–10% FBS were microinjected with MEK-2E DNA (0.4 μg/μl) and rat IgG (2 mg/ml). Six hours after injection, cells were switched to N2 medium and incubated at 39°C for 26 h. (A) Rat IgG (a marker for the injected cells) as detected by Texas red-conjugated goat anti-rat IgG. (B) Phase-contrast micrograph of the same cells. The injected cells are indicated by arrowheads. (C) Phase-contrast micrograph of uninjected Raf-1:ER cells treated with 1 μM estradiol for 26 h. Magnification, ×200. A total of 32 cells were analyzed.

cells within 36 h. Taken together, the data raise the possibility of the existence of an alternative pathway which might involve proteins other than MEK, ERK1, and ERK2 in H19-7 differentiation by Raf-1.

Previous studies have suggested the existence of a MAPK-independent signal transduction pathway for Raf. PC12 cells transfected with the activated *raf-1* oncogene were reported to differentiate (35) without detectable activation of MAPK (36). Activation of MAPK by the oncogenes *ras* and *raf* was observed in NIH 3T3 cells but not in Rat-1a cells (12). Similarly, expression of the ΔRaf-1:ER protein in Rat 1a cells led to activation of MEK by estradiol but no detectable stimulation of ERK1 and -2 (29). In addition, a kinase activity distinct from ERK that is activated by Raf-1 and phosphorylates c-*fos* has recently been described (1). Finally, Raf-1 has recently been shown to activate cdc25, a cell cycle-regulated phosphatase (11). These data suggest that the effects of Raf can be mediated by MEK and/or ERK-independent signal transduction pathways.

The question of whether sustained MAPK activation is sufficient for PC12 cell differentiation is not entirely resolved at present. Microinjection of constitutively activated MEK (6), *Xenopus* MAPKK (10), or MAPK (10) has been shown to induce differentiation of PC12 cells as rapidly as 24 h (6). However, two very recent reports based on expression of Trk (23) and platelet-derived growth factor (34) mutant receptors suggest that activation of the Ras/Raf/MAPK pathway by nerve growth factor or platelet-derived growth factor is not sufficient for differentiation of PC12 cells within 6 days, the normal differentiation time for PC12 cells. One possible explanation for this discrepancy is that superphysiological expression of MEK or MAPK leads to differential regulation of signaling pathways compared with stimulation in response to growth factors.

It is possible to induce extended duration of the MAPK signal and differentiation in PC12 cells by overexpression of the EGF or insulin receptor (6, 32). We have similarly noted that EGF induces many of the same genes as FGF in H19-7 cells (12a), but the level of induction by EGF is significantly decreased, consistent with the suggestion that enhancing the magnitude of the growth factor signal is critical for differentiation. These results clearly implicate magnitude of signal as well as cellular environment in determining physiological outcomes of signaling pathways.

Our results indicate that sustained MAPK activation is not sufficient for differentiation of H19-7 cells within 1 to 2 days of stimulation either in the presence or in the absence of func-

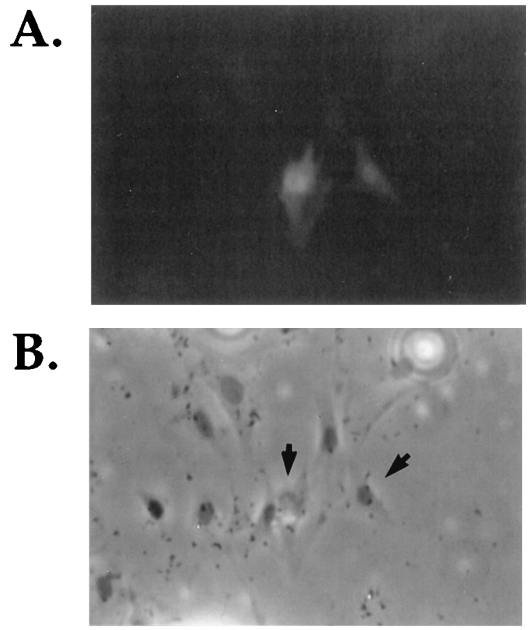

FIG. 13. Microinjection of activated ΔMEK1:ER did not differentiate H19-7 cells. Localization of H19-7 cells injected with ΔMEK1:ER DNA is shown. H19-7 cells in DMEM–10% FBS were microinjected with the plasmid expressing ΔMEK1:ER (0.5 μg/μl), left at 33°C for 6 h, and then switched to N2 medium containing 1 μM estradiol for 48 h at 39°C. (A) Detection of cells expressing ΔMEK1:ER with a monoclonal rat anti-estrogen receptor (H222) and Texas red-labeled goat anti-rat IgG. (B) Phase-contrast micrograph of the same cells. Cells detected with the anti-estrogen receptor antibody are indicated with arrows. Magnification, ×320.

tional large T antigen. The lack of differentiation by Raf within 2 days at 33°C is undoubtedly related to the presence of large T antigen, which may interact with nuclear proteins required for the rapid differentiation response. However, the H19-7 cells, even in the presence of functional T antigen, can be induced to differentiate by activated Raf after a significant lag time. Clearly, constitutive ERK activation is not sufficient to induce this differentiation in a timely fashion, suggesting that another factor that needs to build up over time is required. A factor that is induced by ΔRaf-1:ER and leads to eventual activation of other signaling pathways has also been noted previously (19). It is possible that a similar phenomenon is occurring in PC12 cells, which normally take 6 days to differentiate in response to growth factors. It should be noted that PC12 is a transformed cell line, but in this case the oncogene has not been identified. Therefore, it is possible that PC12 cells, like the H19-7 cells that express functional T antigen, have additional factors that may alter the normal signaling events in the cell.

The observation that MEK and MAPK activation is not sufficient for neuronal differentiation of H19-7 cells suggests that there may be some differences in the differentiation pathways for the hippocampal H19-7 cells and PC12 cells. In H19-7 cells, expression of constitutively activated MEK in H19-7 cells by transfection or microinjection for 36 h was not sufficient to induce differentiation. Furthermore, the differentiation by activated Raf did not appear to result from overactivation of MEK or MAPK. In particular, stimulation of ΔRaf-1:ER at low estradiol concentrations led to limited activation of MEK and MAPK, and the time course of differentiation was similar to that induced by FGF. Since PC12 cells are transformed, it is possible that they express one or more genes that account for the differences observed between the two cell lines when MEK is overexpressed.

The activation state of ΔRaf-1:ER, MEK, and the ERKs in H19-7 cells varied significantly in response to temperature shift or a specific activator, suggesting that phosphatases may play an important regulatory role. At 33°C, the temperature at which cells express functional T antigen, the levels of MEK and MAPK activation were consistently high, implying either that an inhibitor of phosphatases was constitutively expressed (or induced) or that the expression of MAPK phosphatases was repressed. The phosphatase inhibitors okadaic acid and vanadate have been shown to enhance the level of MAPK activation in Raf-stimulated Rat-1a cells (29), and we have observed some activation of MAPK by these phosphatase inhibitors in H19-7 cells (15a). These results are consistent with a hypothesis that large T antigen negatively regulates the activity of cellular phosphatases that may be constitutively expressed or induced by activation of the MAPK cascade. One precedent for such regulation is the inactivation of the phosphatase PP2A by the direct binding of small t antigen (30). While the mechanism by which T antigen may affect phosphatase activity is not known, other possibilities include direct transcriptional regulation of phosphatases or phosphatase inhibitors. One family of dual-specificity phosphatases that act on MAPK and are transcriptionally regulated in response to growth factors is the MKP-1 family (31). The regulation of these and other phosphatases by T antigen is under investigation.

These results illustrate another advantage of the conditionally immortalized H19-7 cell system. The ability to identify and manipulate the immortalization gene encoding large T antigen in the presence or absence of a differentiating signal enables one to assess the effect of the immortalization gene on signaling pathways. In addition, because T antigen is not active at physiological temperatures, it is possible to graft H19-7 cells into rat brains to determine the effect of potential differentiating genes such as *raf-1* in vivo. It will be interesting to compare the effect of constitutively expressed Raf on the differentiation state and survival of these cells in vivo.

The observation that cells expressing functional T antigen can still express neurites in response to Raf-1 activation suggests that the initiation of differentiation is not just a consequence of the cessation of cell growth. Thus, the cells appear to be responding to both a growth-promoting signal (large T antigen) and a differentiating signal (Raf-1), and the ultimate endpoint is presumably determined by the relative potency of the two signals. We have noted that cells expressing v-*raf* constitutively have lower levels of activated Raf than ΔRaf-1:ER cells after induction with estradiol (data not shown); any cells that differentiated in response to a strong constitutive Raf signal would have been lost during selection. A similar proliferation of cells that partially undergo differentiation has also been observed in *Drosophila* mutants (32a). Activation of ΔRaf-1:ER at 33°C with estradiol will also eventually cause neurite formation, but there is a significant lag time consistent with the requirement for an additional factor. Thus, it is possible that Raf uses different pathways for promoting differentiation of neuronal cells depending on the presence or absence of a strong growth-promoting signal.

Whether MEK or ERK activation is required for differentiation of H19-7 cells has not yet been determined. Although MEK and ERK activation levels are low under some conditions of differentiation, it is still possible that only a minimal stimulation is necessary to promote differentiation. Alternatively, there are many other potential signaling factors that could play a role. For example, multiple MEKs and MAPKs have been described in mammalian cells. To date, only three

MAPK-related pathways have been well characterized: the ERK, the JNK/SAPK, and the p38/HOG pathways (reviewed in reference 14). Recent studies have shown that JNK/SAPK and p38/HOG are stress-related kinases that are not significantly activated by tyrosine kinase-activating growth factors or Raf, observations that we have confirmed in studies using H19-7 cells (reference 7, 21, and 37 and data not shown). The nature of the other signaling pathways that mediate the effects of Raf on neuronal differentiation of H19-7 cells are under investigation.

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REFERENCES

1. **Agarwal, S., M. J. Corbley, and T. M. Roberts.** 1995. Reconstitution of signal transduction from the membrane to the nucleus in a baculovirus expression system: activation of Raf-1 leads to hypermodification of c-jun and c-fos via multiple pathways. Oncogene **11:**427–438.
2. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell **42:**841–848.
3. **Chao, T.-S. O., M. K. Abe, M. B. Hershenson, I. Gomes, and M. R. Rosner.** Unpublished observations.
4. **Chao, T.-S. O., K. L. Byron, K.-M. Lee, M. Villereal, and M. R. Rosner.** 1992. Activation of MAP kinase by calcium-dependent and calcium-independent pathways. J. Biol. Chem. **267:**19876–19883.
5. **Chao, T.-S. O., S. A. Qureshi, M. Hendrickson, D. A. Foster, U. Rapp, and M. R. Rosner.** 1994. Differential Raf-1 requirement for activation of MAP kinase by growth factors and tumor promoters. J. Biol. Chem. **269:**7337–7341.
6. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:**841–852.
7. **Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and R. J. Davis.** 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267:**682–685.
8. **Eves, E. M., J. Kwon, M. Downen, M. S. Tucker, B. H. Wainer, and M. R. Rosner.** 1994. Conditional immortalization of neuronal cells from postmitotic cultures and adult CNS. Brain Res. **656:**396–404.
9. **Eves, E. M., M. S. Tucker, J. D. Roback, M. Downen, M. R. Rosner, and B. H. Wainer.** 1992. Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression. Proc. Natl. Acad. Sci. USA **89:**4373–4377.
10. **Fukuda, M., Y. Gotoh, T. Tachibana, K. Dell, S. Hattori, Y. Yoneda, and E. Nishida.** 1995. Induction of neurite outgrowth by MAP kinase in PC12 cells. Oncogene **11:**239–244.
11. **Galaktionov, K., C. Jessus, and D. Beach.** 1995. Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Genes Dev. **9:**1046–1058.
12. **Gallego, C., S. K. Gupta, L. E. Heasley, N.-X. Oian, and G. I. Johnson.** 1992. Mitogen-activated protein kinase activation resulting from selective oncogene expression in NIH 3T3 and Rat 1a cells. Proc. Natl. Acad. Sci. USA **89:**7355–7359.

12a. **Gomes, I., and M. R. Rosner.** Unpublished data.

13. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a norandregenic clonal line of rat adrenal phaeochromocytoma cells that respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73:**2424–2428.
14. **Herskowitz, I.** 1995. MAP kinase pathways in yeast: for mating and more. Cell **80:**187–197.
15. **Kuo, W.-L., B. D. Gehm, and M. R. Rosner.** 1991. Regulation of insulin degradation: expression of an evolutionarily conserved insulin-degrading enzyme increases degradation by an intracellular pathway. Mol. Endocrinol. **5:**1467–1476.

15a. **Kuo, W.-L., J. Rhee, and M. R. Rosner.** Unpublished data.

16. **Lim, K., and C. B. Chae.** 1989. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. BioTechniques **7:**576–579.
17. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn.** 1994. Transformation of mammalian cells by constitutively-active MAP kinase kinase. Science **265:**966–969.
18. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.

18a. **McCarthy, S., H. Cherwinski, M. L. Samuels, and M. McMahon.** Unpublished observations.

18b. **McCarthy, S. A., and M. McMahon.** Unpublished observations.

19. **McCarthy, S. A., M. L. Samuels, C. A. Pritchard, J. A. Abraham, and M. McMahon.** 1995. Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. Genes Dev. **9:**1953–1964.
20. **Meloche, S., G. Pages, and J. Pouyssegur.** 1992. Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44^{mapk}. Mol. Biol. Cell **3:**63–71.
21. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
22. **Noda, M., M. Ko, A. Ogura, D. G. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318:**73–75.
23. **Peng, X., L. A. Greene, D. R. Kaplan, and R. M. Stephens.** 1995. Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. Neuron **15:**395–406.
24. **Rapp, U. R., M. D. Goldsborough, G. E. Mark, T. I. Bonner, J. Groffen, F. H. Reynolds, and J. R. Stephenson.** 1983. Structure and biological activity of v-*raf*, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA **80:**4218–4222.
25. **Robbins, D. J., E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb.** 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J. Biol. Chem. **268:**5097–5106.
26. **Russell, M., S. Winitz, and G. L. Johnson.** 1994. Acetylcholine muscarinic m1 receptor regulation of cyclic AMP synthesis controls growth factor stimulation of Raf activity. Mol. Cell. Biol. **14:**2343–2351.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Samuels, M. L., and M. McMahon.** 1994. Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3T3 cells expressing DRaf-1:ER, an estradiol-regulated form of Raf-1. Mol. Cell. Biol. **14:**7855–7866.
29. **Samuels, M. L., M. J. Weber, J. M. Bishop, and M. McMahon.** 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. Mol. Cell. Biol. **13:**6241–6252.
30. **Sontag, E., S. Fedorov, C. Kamibayashi, D. Robbins, M. Cobb, and M. Mumby.** 1993. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell **75:**887–897.
31. **Sun, H., C. H. Charles, L. F. Lau, and N. K. Tonks.** 1993. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell **75:**487–493.
32. **Traverse, S., K. Seedorf, H. Paterson, C. J. Marshall, P. Cohen, and A. Ullrich.** 1994. EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr. Biol. **4:**694–701.

32a. **Treisman, J.** Personal communication.

33. **Tucker, M. S., E. M. Eves, B. H. Wainer, and M. R. Rosner.** 1993. Activation of MAP kinase by epidermal growth factor in hippocampal neurons and neuronal cell lines. J. Neurochem. **61:**1376–1387.
34. **Vaillancourt, R. R., L. E. Heasley, J. Zamarripa, B. Storey, M. Valius, A. Kazlauskas, and G. L. Johnson.** 1995. Mitogen-activated protein kinase activation is insufficient for growth factor receptor-mediated PC12 cell differentiation. Mol. Cell. Biol. **15:**3644–3653.
35. **Wood, K. W., H. Qi, G. D'Arcangelo, R. C. Armstrong, T. M. Roberts, and S. Halegoua.** 1993. The cytoplasmic *raf* oncogene induces a neuronal phenotype in PC12 cells: a potential role for cellular *raf* kinases in neuronal growth factor signal transduction. Proc. Natl. Acad. Sci. USA **90:**5016–5020.
36. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell **68:**1041–1050.
37. **Yan, M., T. Dai, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton.** 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature (London) **372:**798–800.
38. **Yan, M., and D. J. Templeton.** 1994. Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. J. Biol. Chem. **269:**19067–19073.