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Differentiation of Central Nervous System Neuronal Cells by Fibroblast-Derived Growth Factor Requires at Least Two Signaling Pathways: Roles for Ras and Src

WEN-LIANG KUO, KWANG-CHUL CHUNG, AND MARSHA RICH ROSNER*

Ben May Institute for Cancer Research and Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637

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To evaluate the role of mitogen-activated protein (MAP) kinase and other signaling pathways in neuronal cell differentiation by basic fibroblast-derived growth factor (bFGF), we used a conditionally immortalized cell line from rat hippocampal neurons (H19-7). Previous studies have shown that activation of MAP kinase kinase (MEK) is insufficient to induce neuronal differentiation of H19-7 cells. To test the requirement for MEK and MAP kinase (ERK1 and ERK2), H19-7 cells were treated with the MEK inhibitor PD098059. Although the MEK inhibitor blocked the induction of differentiation by constitutively activated Raf, the H19-7 cells still underwent differentiation by bFGF. These results suggest that an alternative pathway is utilized by bFGF for differentiation of the hippocampal neuronal cells. Expression in the H19-7 cells of a dominant-negative Ras (N17-Ras) or Raf (C4-Raf) blocked differentiation by bFGF, suggesting that Ras and probably Raf are required. Expression of dominant-negative Src (pcSrc295Arg) or microinjection of an anti-Src antibody blocked differentiation by bFGF in H19-7 cells, indicating that bFGF also signals through a Src kinase-mediated pathway. Although neither constitutively activated MEK (MEK-2E) nor v-Src was sufficient individually to differentiate the H19-7 cells, coexpression of constitutively activated MEK and v-Src induced neurite outgrowth. These results suggest that (i) activation of MAP kinase (ERK1 and ERK2) is neither necessary nor sufficient for differentiation by bFGF; (ii) activation of Src kinases is necessary but not sufficient for differentiation by bFGF; and (iii) differentiation of H19-7 neuronal cells by bFGF requires at least two signaling pathways activated by Ras and Src.

Growth factor-stimulated tyrosine kinase receptors such as the basic fibroblast-derived growth factor (bFGF) receptor activate a number of discrete signaling pathways that can lead to either cell growth or differentiation depending on the particular system. There are at least two possible explanations for the differences in the downstream cellular responses: differences in the amplitude, duration, or localization of a particular signal, and differences in the key signaling pathways and cellular targets. While it is likely that both factors play a role in determining the final outcome, a more general understanding of the process requires identification of the key signaling pathways that lead to a particular end point.

Mitogen-activated protein (MAP) kinase is one mediator that is activated by a wide variety of growth factors and other effectors and acts as a transducer of signals from the cell surface to the nucleus. Upon binding of growth factors, receptor tyrosine kinases initiate a cascade of events involving activation of Ras, which stimulates a kinase cascade consisting of Raf, MAP kinase kinase (MEK), and MAP kinase. Once activated, MAP kinase translocates to the nucleus, where it phosphorylates transcription factors leading to events such as growth and differentiation. Thus, MAP kinase has been proposed as a central mediator of both the growth and differentiation processes in a number of tissues including those of the central nervous system (CNS).

Since primary neural progenitor cells are heterogeneous, it is advantageous to initially test the role of MAP kinase in the differentiation of neuronal cells by using a clonal model system. PC12, a tumor cell line derived from rat adrenal pheochromocytomas, has been a widely used cell line for studying signaling pathways leading to peripheral nervous system neuronal differentiation in response to extracellular stimuli such as nerve growth factor. The observation that factors, such as NGF and bFGF, that induce PC12 cell differentiation also stimulate prolonged MAP kinase activation, in conjunction with studies involving constitutive activation or inhibition of MAP kinase, led to the hypothesis that sustained activation of MAP kinase is necessary and sufficient for neuronal differentiation (9, 30, 33). More recently, studies with various mutant receptors for differentiation of PC12 cells suggested that receptor stimulation of the Ras/Raf/MAP kinase pathway alone was insufficient to induce PC12 cell differentiation (31, 45). Some studies with PC12 cells have concluded that activation of Src is necessary and sufficient for neuronal differentiation by tyrosine kinase receptors (10), whereas other studies propose alternative mechanisms (45). To resolve these apparent contradictions and determine the generality of these observations, it is important to investigate neuronal cell culture systems other than PC12.

As a model system for studying the differentiation of neuronal progenitor cells from the CNS, we have generated a conditionally immortalized hippocampal cell line (H19-7) by transducing rat E17 hippocampal cells with a retroviral vector expressing a temperature-sensitive simian virus 40 (SV40) large T antigen (14). The H19-7 cells proliferate in response to epidermal growth factor at the permissive temperature in the presence of active T and differentiate in response to bFGF at the nonpermissive temperature when T is inactivated. The differentiated H19-7 cells do not divide in response to serum,

* Corresponding author. Mailing address: Ben May Institute for Cancer Research, University of Chicago, 5841 S. Maryland Ave., MC 6027, Chicago, IL 60637. Phone: (773) 702-0380. Fax: (773) 702-4634. E-mail: mrosner@ben-may.bsd.uchicago.edu.

extend neurites and undergo morphological changes, express neuronal markers such as neurofilaments and brain type II sodium channels, and display action potentials (13, 14, 22). The ability of the conditionally immortalized rat hippocampal neuronal progenitor line H19-7 to differentiate in response to bFGF mimics the response of primary hippocampal cells and selected other neuronal cell types during late embryogenesis, since bFGF can act as a differentiating factor in certain regions of the CNS expressing the bFGF receptor, such as the hippocampus and the cortex (35, 47). Neuronal cell lines that have been similarly immortalized with temperature-sensitive large T antigen have the capability of developing into region-specific neurons upon transplantation (36, 42), indicating that the transient expression of large T antigen creates cell lines that are able to respond in vivo to environmental cues. In contrast to neuroblastomas or cell lines like PC12, it is possible to study signaling pathways in the H19-7 cells in the absence of immortalizing or transforming genes.

In a previous study, we have shown that constitutively activated Raf is sufficient to differentiate H19-7 cells (20). As in PC12 cells, cellular differentiation of H19-7 cells by bFGF correlates with prolonged MAP kinase activation (20). However, in contrast to PC12 cells, constitutive activation of neither MAP kinase nor MEK is sufficient to induce the differentiation of H19-7 cells. These results suggest that the constitutively active Raf stimulates downstream targets other than MEK that are required for Raf-mediated differentiation.

In this study, we sought to determine whether activation of the MAP kinase signaling pathway is required for differentiation of H19-7 cells and to identify other key signaling pathways. A specific inhibitor of MEK, PD098059, and dominant-negative mutants of Ras, Raf, and Src were used to probe the signaling molecules that are essential in the differentiation process. The results indicate that differentiation by Raf-1 kinase requires the activation of MAP kinase (ERK1 and ERK2), but differentiation by the bFGF receptor kinase may occur without MAP kinase activation. This result raised the possibility that the bFGF receptor kinase activates an alternative pathway, and we showed that both Src- and Ras-stimulated pathways are required for bFGF-induced neuronal differentiation. Finally, although v-Src or MEK alone was not sufficient for differentiation of hippocampal neurons, the combination of v-Src and constitutively active MEK promoted neurite outgrowth.

MATERIALS AND METHODS

Materials. Protein A-Sepharose was purchased from Pharmacia. Fetal bovine serum (FBS), Dulbecco's minimal essential medium (DMEM), and Geneticin were purchased from Life Technologies Inc. Myelin basic protein (MBP), hygromycin, muscle enolase, and estradiol were purchased from Sigma. Human bFGF (receptor grade) was purchased from Bachem. Buffered Formalde-Fresh (10% formalin) was purchased from Fisher Scientific (Fair Lawn, N.J.). Monoclonal antibody against the hemagglutinin (HA) epitope (12CA5) was purchased from BabCo. Antibodies against neurofilament proteins 68 were purchased from Sigma. The plasmid encoding the mutant Ras (N17-Ras) was a gift from L. Feig. The plasmid encoding mutant Raf (C4-Raf) was a gift from U. Rapp. The plasmid encoding mutant Src (pcSrc295Arg) was a gift from D. Foster, and v-Src (pMvSrc) and a control plasmid (pEVX) were gifts from D. Shalloway (19). Plasmids expressing constitutively active MEK1 (MEK-2E) protein and nonexpressing plasmid EE-CMV were gifts from D. Templeton (49). PD098059 was a gift from A. Saltiel. The rabbit polyclonal anti-Src antibody (cst-1) was a gift from S. Courtneidge. The cytomegalovirus–β-galactosidase expression vector was a gift from V. Sukhatme. Plasmid DNAs were prepared by CsCl-ethidium bromide gradient centrifugation as described previously (21). The 1% Triton-based lysis buffer (TLB) contains 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM disodium EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 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 pyrophosphate, 1 mM β-glycerophosphate (pH 7.4), and 0.1 g of *p*-nitrophenylphosphate per ml.

Cells. H19-7 cells were generated from rat hippocampal neurons (14). They were conditionally immortalized by stable transfection with temperature-sensitive SV40 large T antigen. They were grown in DMEM plus 10% FBS and maintained at 33°C under G418 selection throughout the experiments (14). To induce differentiation, the cells were placed in N2 medium and shiftd to 39°C prior to treatment with differentiating agents as previously described (14). Differentiated cells were defined as cells with a rounded and refractile cell body containing at least one neurite whose length is greater than the diameter of the cell body.

ΔRaf-1:ER cells (20) were generated by stably transfecting H19-7 cells (14) with the vector expressing the estradiol-regulated human oncogenic Raf-1 (ΔRaf-estrogen receptor fusion protein [ΔRaf-1:ER]) (41).

Effect of the MEK inhibitor, PD098059, on HA-ERK2 or endogenous MAP kinase activity. For assaying ectopically expressed HA-ERK2, H19-7 cells were plated on poly-L-lysine-coated plates and grown in DMEM–10% FBS overnight, and then HA-ERK2 DNA (10 μg/plate) was transfected into the cells by the calcium phosphate method (39). About 36 h before being harvested, the cells were switched to N2 medium and shifted to 39°C. The MEK inhibitor was added 15 min before the induction of bFGF, and the cells were lysed in 1% Triton-based lysis buffer. HA-ERK2 protein was immunoprecipitated and assayed for kinase activity by MBP phosphorylation in vitro as described previously (20).

For assaying the endogenous MAP kinases in ΔRaf-1:ER cells, the cells were plated in six-well dishes at a density of 10^5 cells per well. The next day, the cells were serum starved in N2 medium and shifted to 39°C for 24 h. Subsequently, the cells were left untreated or treated with the MEK inhibitor for 15 min and bFGF (6 to 10 ng/ml) or estradiol (1 μM) was added for different lengths of time. The cells were lysed in 300 μl of 1% Tris-based lysis buffer (see Materials and Methods), and 50 to 100 μg of cellular proteins was used for purification of endogenous MAP kinases with polyclonal antibodies against both ERK1 and ERK2 (40). MAP kinase activity was assayed with MBP as a substrate in the presence of [γ-^{32}P]ATP as described previously (20).

Effect of PD098059 on differentiation of the hippocampal cell lines. ΔRaf-1:ER or H19-7 cells were plated and grown on poly-L-lysine-coated 12-well dishes until the cells reached 50 to 60% confluence. The cells were switched to N2 medium, treated with PD098059 at a final concentration of 20 μM for 15 min, and stimulated with bFGF (6 to 10 ng/ml) or estradiol (1 μM). The treated cells were continuously cultured at 39°C for 36 to 48 h, analyzed for morphological changes with a Zeiss microscope, and photographed with a 35-mm Nikon camera.

Immunofluorescent staining of neurofilament protein 68. ΔRaf-1:ER cells were grown on poly-L-lysine-coated coverslips in DMEM containing 10% bovine serum at 33°C. The next day, the cells were switched to N2 medium and cultured at 39°C. They were treated with the MEK inhibitor for 15 min and stimulated with bFGF or estradiol at final concentrations of 6 ng/ml and 1 μM, respectively. At 36 to 48 h after the induction, the cells were fixed in 10% buffered Formalde-Fresh for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in 2% formalin for 5 min, and blocked with goat serum. Monoclonal antibodies against neurofilament protein 68 were added at a 1:200 dilution, and the mixture was incubated for 1 h at room temperature. The cells were extensively washed with phosphate-buffered saline, and a secondary antibody, fluorescein-conjugated anti-mouse immunoglobulin G (IgG), was added at a 1:300 dilution and incubated for 1 h at room temperature. The cells were extensively washed with phosphate-buffered saline, and the coverslips were mounted on slides with Vectashield (Vector Laboratories, Inc., Burlingame, Calif.). The cells were analyzed with an immunofluorescence microscope, and the pictures were printed with a Sony Video Printer.

Microinjection of H19-7 cells. About 4×10^4 cells were seeded on poly-L-lysine-coated coverslips placed in 12-well dishes. After the cells grew to the desired densities, the cells were comicroinjected with injection buffer (50 mM HEPES [pH 7.4], 40 mM NaCl) containing rabbit polyclonal Src antibody (cst-1; 4 μg/μl) and rat IgG (1 μg/μl) as an antibody marker for injected cells. Microinjection was done with an Eppendorf 5171 micromanipulator, a 5246 microinjector, and a Leitz DMIRB microscope. The cells were analyzed by immunofluorescence. The pictures were printed with a Sony Video Printer.

Transient transfection of H19-7 and ΔRaf-1:ER cells. About 10^5 H19-7 or ΔRaf-1:ER cells were seeded in each well of a six-well plate. The next day, the cells in each well were cotransfected with 2 μg of test or control vector and 1 μg of a pCMV–β-gal expression vector with TransIT-LT1 solution (PanVera Co., Madison, Wis.). At 12 to 18 h after transfection, the 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 36 to 48 h after the temperature shift and the treatment, the cells were fixed and probed sequentially with an anti-β-galactosidase antibody and a fluorescein-conjugated secondary antibody. Alternatively, the cells were fixed and stained for β-galactosidase activity (24). Cells expressing the β-galactosidase plasmid were categorized for differentiation state according to their morphologies and the length of their processes. Pictures were taken with a Nikon microscope and a Nikon 35-mm camera.

Src kinase assay. H19-7 and NIH 3T3 cells were grown in DMEM containing 10% FBS and then switched to N2 at 39°C or DMEM containing 0.1% FBS at 37°C, respectively, 48 h before being harvested. The H19-7 cells were treated with bFGF (10 ng/ml) for 10 min and the NIH 3T3 cells were treated with

platelet-derived growth factor (PDGF) at 50 ng/ml for 30 min before they were harvested and lysed in RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 7.5), 150 mM NaCl, 20 μg of aprotinin per ml, 200 μM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Then 200 μg of protein was incubated at 4°C for 3 h with the mouse monoclonal anti-Src antibody (MAb327; Oncogene) and protein A-Sepharose precoated with rat anti-mouse IgG. Immunocomplexes were washed three times in RIPA and once in TBS buffer (20 mM Tris [pH 7.5], 150 mM NaCl), and the reactions were carried out at 30°C for 30 min in 20 μl of buffer containing 20 mM HEPES (pH 7.2), 5 mM $MnCl_2$, 200 μM sodium orthovanadate, 5 μg of acid-treated enolase, 10 μM ATP, and 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol). The reactions were stopped by adding 4× Laemmli buffer and boiling for 3 min, and phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Assay of CAT in transiently transfected H19-7 cells. About 5×10^5 H19-7 cells were seeded in 100-mm dishes. The next day, the cells were cotransfected with either a v-Src expression vector (pMvSrc) or the control vector (pEVX) and both pCMV–β-gal and p667, a mammalian expression vector containing the truncated Egr-1 promoter (pE425/+65) linked to chloramphenicol acetyltransferase (CAT) cDNA (34). At 24 h after transfection, the cells were switched to N2 medium at 39°C and treated or not treated with bFGF for 24 h before being harvested. CAT expression was assayed by an enzyme-linked immunosorbent assay (5′-3′ Inc.). The level of CAT expression was normalized to the activity of β-galactosidase as a control for transfection efficiency.

RESULTS

Activation of MAP kinase by bFGF is blocked by an MEK inhibitor, PD098059. To examine the requirement for MAP kinase activation in neuronal differentiation, a synthetic MEK inhibitor, PD098059, which efficiently and specifically suppresses MAP kinase activation induced by various reagents (1), was used. Initially, we determined the dose response and time course for suppression of MAP kinase activation by the MEK inhibitor in H19-7 cells, a conditionally immortalized cell line from rat hippocampal neurons that expresses a temperature-sensitive SV40 large T antigen (14). In these cells, treatment with bFGF (6 to 10 ng/ml) in N2 medium at 39°C, the temperature at which large T antigen is nonfunctional, results in rapid activation of MAP kinase and subsequent differentiation (20). To determine the effect of the MEK inhibitor on MAP kinase stimulation by FGF at 39°C, MAP kinase activity was measured by phosphorylation of MBP with immunoprecipitated HA-tagged ERK2 that had been transiently transfected into H19-7 cells. The cells were pretreated with the MEK inhibitor for 10 min or left untreated and then stimulated with 10 nM bFGF for 10 min, the time of maximal ERK activity. The dose-response curve showed that concentrations of the MEK inhibitor of ≥20 μM reduced the phosphorylation of MBP to a level lower than the basal level and comparable to that from nonspecific kinase activity in mock-transfected cells (Fig. 1). Similar results were obtained with ΔRaf-1:ER cells, which are H19-7 cells that express an oncogenic Raf kinase fused to the ligand binding domain of the estrogen receptor (20, 22). On the basis of these results, we concluded that 20 μM MEK inhibitor was sufficient to suppress bFGF-stimulated MAP kinase activity in H19-7 cells.

To ensure that the effect of the MEK inhibitor was sustained, we determined the effect of PD098059 on the time course of MAP kinase activation by either bFGF or Raf-1 kinase. For this experiment, we used ΔRaf-1:ER cells, which undergo MAP kinase activation and differentiation in response to either bFGF- or estradiol-induced activation of the ΔRaf-1:ER fusion protein. In this case, endogenous MAP kinase was immunoprecipitated with an anti-MAP kinase antibody and assayed for MBP phosphorylation. The results showed that stimulation of MAP kinase activity by bFGF was blocked by 20 μM MEK inhibitor for at least 24 h (Fig. 2A [left] and B). In these experiments, the level of nonspecific kinase activity, assessed by immunoprecipitation of extract from bFGF-stimulated cells with protein A beads in the absence of antibody, was

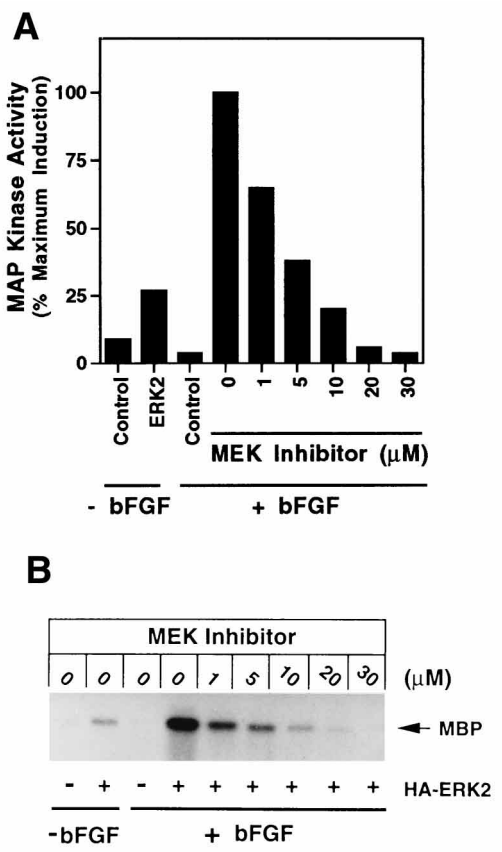

FIG. 1. Dose response for the inhibition of MAP kinase activity by the MEK inhibitor PD098059. H19-7 cells were mock transfected with vector alone or transfected with pHA-ERK2 DNA by the calcium phosphate procedure as described in Materials and Methods. About 48 h before being harvested, the transfected cells were treated with PD098059 at various concentrations for 10 min and then stimulated with bFGF (10 ng/ml) for 10 min. HA-ERK2 protein was immunoprecipitated from the transfected cells with an HA-specific antibody, 12CA5, and assayed for kinase activity with MBP as a substrate. The reaction mixtures were resolved by SDS-PAGE and then visualized by autoradiography. (A) Graph depicting the dose response for inhibition of MAP kinase activity by PD098059. [^{32}P]MBP bands in the autoradiograph (B) were quantitated with an AMBIS image system. Maximum induction (100%) is the level of MAP kinase activity in the cells transfected with HA-ERK2 DNA and treated with bFGF, in the absence of PD098059. The control samples were from mock-transfected cells. (B) Autoradiograph showing MBP phosphorylation from the samples in panel A. MBP bands are indicated with an arrow. The results are representative of two independent experiments.

the same as the basal level of MBP phosphorylation observed in anti-MAP kinase immunoprecipitates from unstimulated cell extracts (22). Pretreatment of these cells with 20 μM MEK inhibitor also suppressed the induction of MAP kinase by the estradiol-activated ΔRaf-1:ER kinase for up to 24 h (Fig. 2A [right] and C). Similar results were obtained when 30 μM MEK inhibitor was used (22).

MAP kinase activation is required for Raf-1-induced but not bFGF-induced differentiation. Since the MEK inhibitor could efficiently suppress the activation of MAP kinases, we used it to test the requirement for MAP kinase activation in the differentiation of hippocampal neurons induced by either bFGF or activated Raf-1 kinase. ΔRaf-1:ER cells were pretreated with 20 μM MEK inhibitor or left untreated for 15 min before the addition of 6 nM bFGF or 1 μM estradiol. At about 36 h after treatment, the cells were analyzed for differentiation by the criteria of morphological change, neurite extension, and neu-

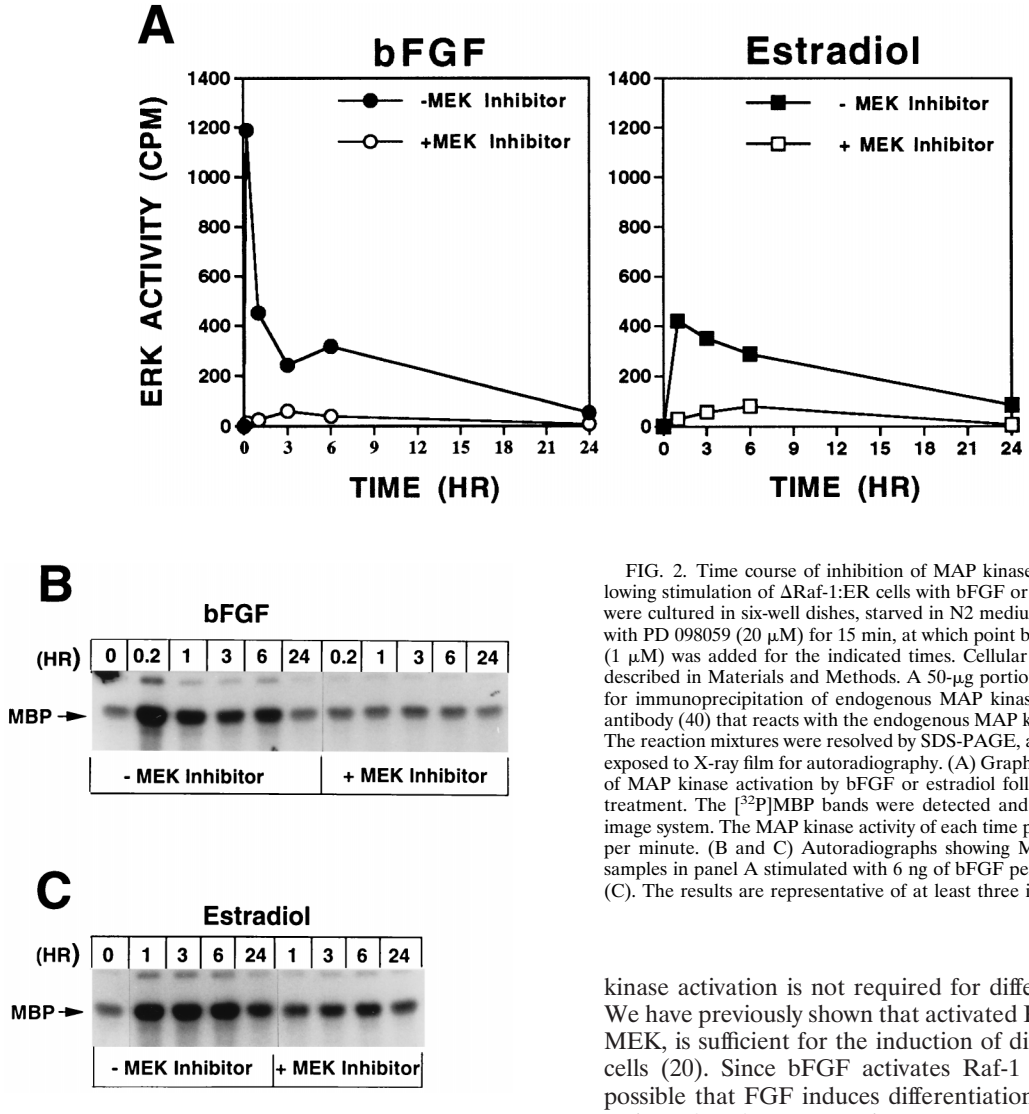

FIG. 2. Time course of inhibition of MAP kinase activity by PD098059 following stimulation of ΔRaf-1:ER cells with bFGF or estradiol. ΔRaf-1:ER cells were cultured in six-well dishes, starved in N2 medium for 24 h, and pretreated with PD 098059 (20 μM) for 15 min, at which point bFGF (6 ng/ml) or estradiol (1 μM) was added for the indicated times. Cellular extracts were prepared as described in Materials and Methods. A 50-μg portion of each sample was used for immunoprecipitation of endogenous MAP kinase with a rabbit polyclonal antibody (40) that reacts with the endogenous MAP kinases (ERK1 and ERK2). The reaction mixtures were resolved by SDS-PAGE, and the gels were dried and exposed to X-ray film for autoradiography. (A) Graphs depicting the time course of MAP kinase activation by bFGF or estradiol following MEK inhibitor pretreatment. The [^{32}P]MBP bands were detected and analyzed with an AMBIS image system. The MAP kinase activity of each time point is shown as net counts per minute. (B and C) Autoradiographs showing MBP phosphorylation from samples in panel A stimulated with 6 ng of bFGF per ml (B) or 1 μM estradiol (C). The results are representative of at least three independent experiments.

rofilament staining. Differentiated neurons are characterized by a rounded and refractile cell body containing at least one neurite that is longer than the diameter of the cell body; undifferentiated cells are flat with no extended neurites (20). The effect of the MEK inhibitor on differentiation is shown in Fig. 3. Differentiation induced by activated Raf-1 kinase was blocked by 20 μM MEK inhibitor (Fig. 3B and E), whereas differentiation induced by bFGF was not (Fig. 3C and F). In addition, MEK inhibitor alone did not significantly change the morphology of the cells (Fig. 3A and D). Similar results were obtained for H19-7 cells induced by bFGF (22). To confirm that ΔRaf-1:ER cells still underwent differentiation in response to bFGF despite inhibition of MEK and MAP kinase, we analyzed the cells by immunofluorescence cytochemistry with an antibody against neurofilament protein 68 (NF 68), an intermediate-molecular-weight neurofilament. Increasing the expression of intermediate- and high-molecular-weight neurofilaments is a marker of neuronal differentiation (11). In the presence of 20 μM PD098059, the cells treated with bFGF (Fig. 4B and D) showed an increase in NF 68 staining compared to undifferentiated cells whereas cells treated with estradiol did not (Fig. 4A and C).

Taken together, our results suggest that MEK and MAP kinase activation is not required for differentiation by bFGF. We have previously shown that activated Raf, but not activated MEK, is sufficient for the induction of differentiation in these cells (20). Since bFGF activates Raf-1 in H19-7 cells, it is possible that FGF induces differentiation solely through activation of Raf. However, in contrast to Raf, bFGF-mediated differentiation does not require activation of MEK or MAP kinase. Thus, these results suggest that an alternative pathway is utilized by bFGF for differentiation of hippocampal neuronal cells.

A Src family kinase is required for bFGF-induced differentiation of hippocampal cells. We examined Src as a possible alternative pathway used by bFGF for neuronal differentiation. Src has been reported to associate with bFGF receptors (50). Furthermore, Src family kinases are required for growth factors such as bFGF and PDGF to induce the proliferation and differentiation of a wide variety of cells (4, 6). To test whether activation of Src family kinases might be required for bFGF-induced differentiation, we initially determined whether Src is activated by bFGF in H19-7 cells. Using muscle enolase as a substrate for assaying Src kinase activity in vitro, we detected a small but consistent induction (1.7 ± 0.4-fold) under differentiating conditions. A similar level of induction of Src kinase activity was observed upon PDGF stimulation of NIH 3T3 cells (22), a cell type in which Src has been implicated as a mediator of growth (4).

To block the activation of Src, we initially used a dominant-negative Src (pcSrc295Arg) construct (29) as an inhibitor. The β-galactosidase gene was used as a marker for the transfected

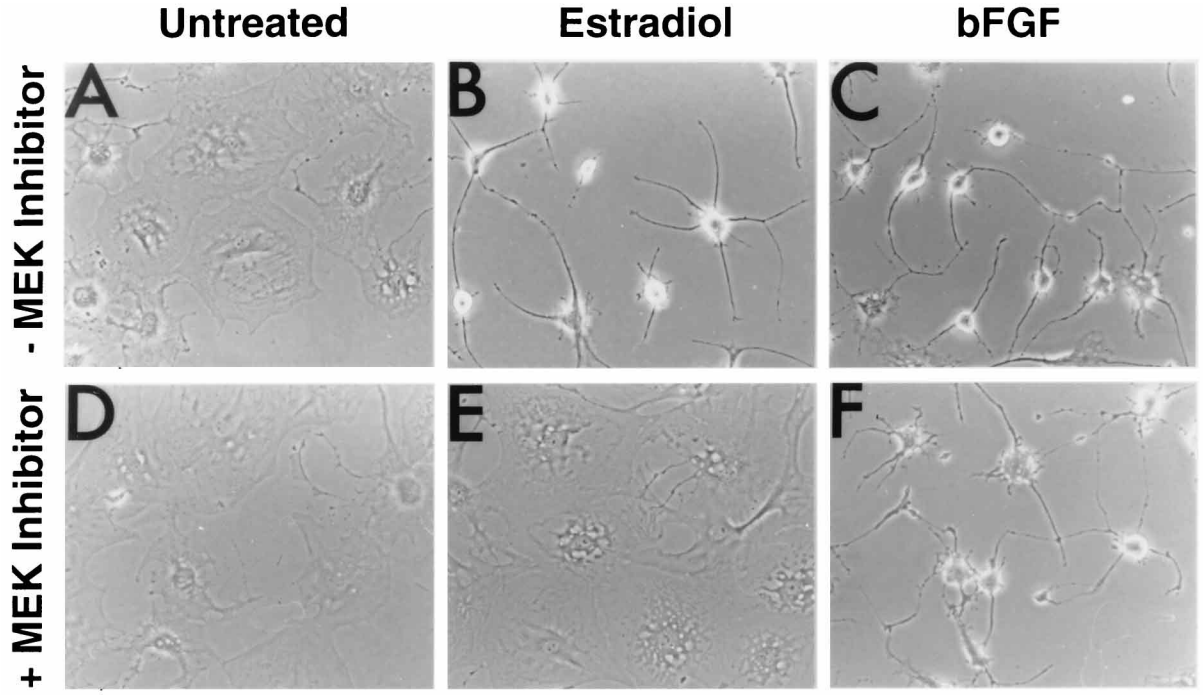

FIG. 3. Effect of the MEK inhibitor PD098059 on differentiation of ΔRaf-1:ER cells induced by bFGF or estradiol. Raf-1:ER cells were grown in 12-well dishes, starved in N2 medium for 24 h, and then left untreated (A to C) or treated (D to F) with 20 μM PD098059 before the addition of the inducing agent, bFGF or estradiol. (A and D) Untreated cells; (B and E) cells stimulated with estradiol; (C and F) cells stimulated with bFGF. At 36 to 48 h after stimulation, cells were visualized by phase-contrast microscopy. Magnification, ×200. The results are representative of more than three independent experiments.

cells. pcSrc295Arg or the vector control (pEVX) was cotransfected along with pCMV–β-gal into H19-7 cells. The cells were treated with bFGF for 36 to 48 hours at 39°C and then analyzed for β-galactosidase-positive cells (Fig. 5). Previously, we determined that the efficiency for expressing cotransfected plasmids in the same cell is about 80% (20). The criteria for evaluating the differentiation state of the neuronal cells are described above. The results show that the untransfected (background) cells that do not express β-galactosidase in both transfection experiments have similar percentages of differentiated cells (~70%). However, cells that express β-galactosidase in the pcSrc295Arg-transfected population had only 22% differentiated cells, in contrast to 65% differentiated cells in the pEVX-transfected population. Similar results were obtained when ΔRaf-1:ER cells were used (22). In contrast, treatment of cells with pcSrc295Arg or an inhibitor of Src family kinases did not significantly block Raf-mediated differentiation of the ΔRaf-1:ER cells (22), suggesting that Src and Raf activate distinct pathways. These results indicate that dominant-negative Src significantly blocked bFGF-induced differentiation of the hippocampal cells.

To confirm these results, we microinjected cst-1 (an antibody that recognizes and inhibits Src, Fyn, and Yes) along with an antibody marker (rat IgG) into H19-7 cells and treated them with bFGF under differentiating conditions for 48 h. As illustrated in Fig. 6, 85% of the cells expressing the antibody (indicated by arrows) were flat with at most short projections, characteristic of undifferentiated cells. In contrast, 87% of uninjected background cells were differentiated.

To determine whether the dominant-negative Src may be acting by interfering with the MAP kinase activation pathway, we cotransfected increasing amounts of pcSrc295Arg along with a plasmid expressing HA-tagged ERK2 into ΔRaf-1:ER cells. The cells were treated for 10 min with bFGF under differentiating conditions, and the HA-ERK2 activity was measured by phosphorylation of MBP with HA-ERK2 immunoprecipitated with anti-HA antibody. The data indicate that the dominant-negative Src did not interfere with the activation of MAP kinase by bFGF (Fig. 7). Taken together, these results suggest that the Src kinase signaling pathway is required for induction of neuronal differentiation by bFGF and is distinct from the MAP kinase signaling pathway.

MEK and Src promote neurite outgrowth in hippocampal neurons. Although Src may be a key mediator of the bFGF signal, it is not clear whether activated Src (e.g., v-Src), like activated Raf, is sufficient to induce differentiation. Before addressing this question, we determined whether transfected v-Src is functionally active in the hippocampal cell lines by initially testing its ability to activate ERK2. pMv-Src, a v-Src-expressing construct, or a control vector (pEVX) was cotransfected with HA-tagged ERK2 into H19-7 cells. The cells were either kept at 33°C, the temperature at which large T antigen is functional, or shifted to 39°C before analysis. At the permissive temperature (33°C), v-Src induced ERK2 activity twofold (Fig. 8A, compare lanes 1 and 2 with lanes 3 and 4). The ERK activation by v-Src kinase was much weaker than the induction by bFGF (Fig. 8A, lanes 5 and 6), indicating that bFGF can also activate ERK by other pathways. No significant stimulation of ERK2 by v-Src was detected at 39°C, the differentiating temperature (22), consistent with the inability of the dominant-negative Src to inhibit ERK induction by bFGF at this temperature (see above). We also tested the ability of v-Src to induce transcription of the CAT gene linked to four serum response elements derived from Egr-1 (p667) that have previously been shown to respond to v-Src (34). v-Src or a control vector (pEVX), along with both p667 and pCMV–β-gal, was

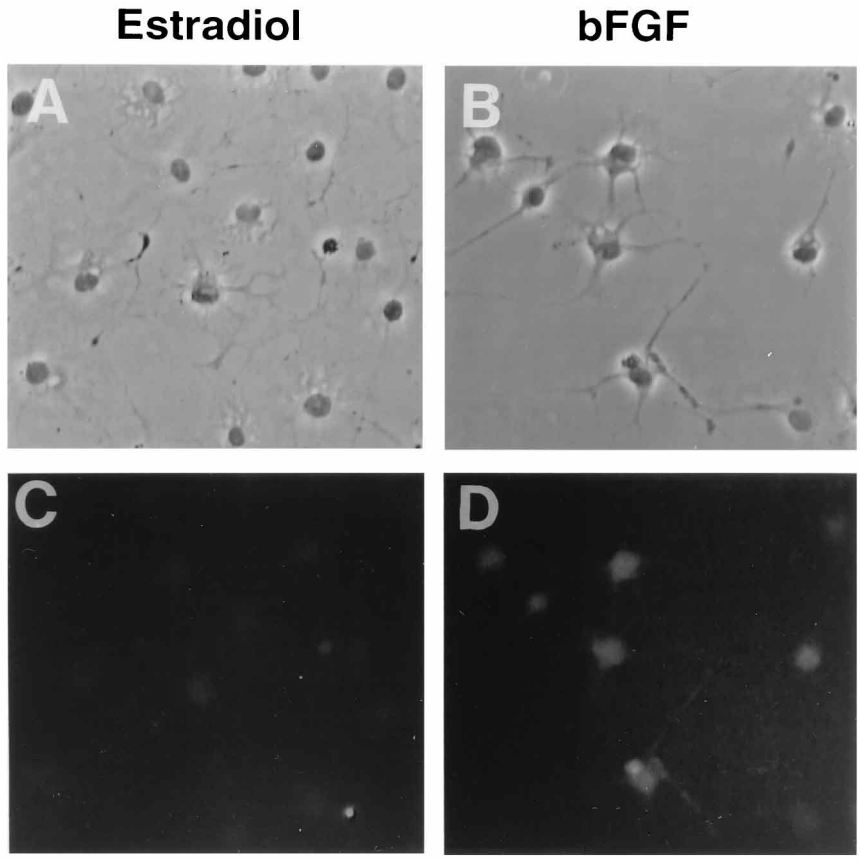

FIG. 4. Expression of NF 68 in ΔRaf-1:ER cells stimulated with bFGF or estradiol. ΔRaf-1:ER cells were grown on poly-L-lysine-coated coverslips in DMEM–10% FBS at 33°C overnight and then switched to N2 medium and shifted to 39°C. The cells were treated with PD098059 for 15 min, and bFGF or estradiol was added. Two days after stimulation, the cells were fixed in formaldehyde, probed with a monoclonal antibody against NF 68, and detected with fluorescein isothiocyanate-conjugated secondary antibody. The same field of cells stimulated with estradiol is shown by phase-contrast microscopy (A) and immunofluorescence microscopy (C). A second field of cells stimulated with bFGF is shown by phase-contrast microscopy (B) and immunofluorescence microscopy (D). Magnification, ×200.

cotransfected into H19-7 cells. Cells were shifted to the nonpermissive temperature (39°C) and then analyzed for CAT activity. As shown in Fig. 8B, CAT activity was induced twofold. These results indicate that v-Src transfected into H19-7 cells is active.

To determine whether v-Src expression could induce hippocampal cell differentiation, we cotransfected β-galactosidase along with either v-Src, a vector control (pEVX), or constitutively activated MEK (MEK2E) into H19-7 cells, transferred the cells to 39°C for 48 h, and analyzed them for morphological changes. The MEK2E-transfected cells were not differentiated, as observed previously (20). Similarly, v-Src-transfected cells remained largely undifferentiated (Fig. 9A). Comparable results were obtained with H19-7 cells that had been given microinjections of v-Src (22). Thus, the expression of v-Src alone is insufficient to differentiate the H19-7 cells.

Although neither constitutively activated MEK (20) nor oncogenic Src could individually differentiate the hippocampal cells, it is possible that signals from these two molecules have an additive or synergistic effect on hippocampal differentiation. To test this possibility, we cotransfected plasmids expressing β-galactosidase, v-Src, and constitutively active MEK (MEK-2E) (49) into H19-7 cells, transferred the cells to 39°C for 48 h, and then analyzed them for changes in cell morphology. In contrast to the cells transfected with the control vector, which remained undifferentiated (Fig. 9A to C), 33% of the cells transfected with MEK-2E and v-Src that expressed β-galactosidase (Fig. 9A, D, and E) had neurite outgrowth but lacked the rounded and refractile cell bodies characteristic of differentiated H19-7 cells. The results suggest that MEK and v-Src represent two discrete signaling pathways that have the potential, when both are constitutively activated, to induce neurite outgrowth in the H19-7 hippocampal cells.

Dominant-negative Ras and dominant-negative Raf block bFGF-induced differentiation of hippocampal neuronal cells. Constitutively activated MEK, in conjunction with v-Src, appears to mimic the action of other signaling pathways stimulated by bFGF, since activation of MEK is not required for differentiation by bFGF. One possibility is that the upstream activators Ras and Raf stimulate these other signaling pathways. To address this question, we introduced dominant-negative Ras (N17-Ras), dominant-negative Raf (C4-Raf), or a control plasmid and β-galactosidase-expressing vectors into the H19-7 hippocampal neuronal cells by transient transfection. As shown in Fig. 10, bFGF induced about 65% of the β-galactosidase-positive cells cotransfected with the control plasmid to differentiate, similar to the β-galactosidase-negative cells on the same plate (22). However, bFGF induced only 12 and 24% of the β-galactosidase-positive cells cotransfected with N17-Ras and C4-Raf, respectively, to differentiate (Fig. 10). Thus, expression of N17-Ras or C4-Raf inhibits differentiation by about 82 and 63% respectively. Similar data

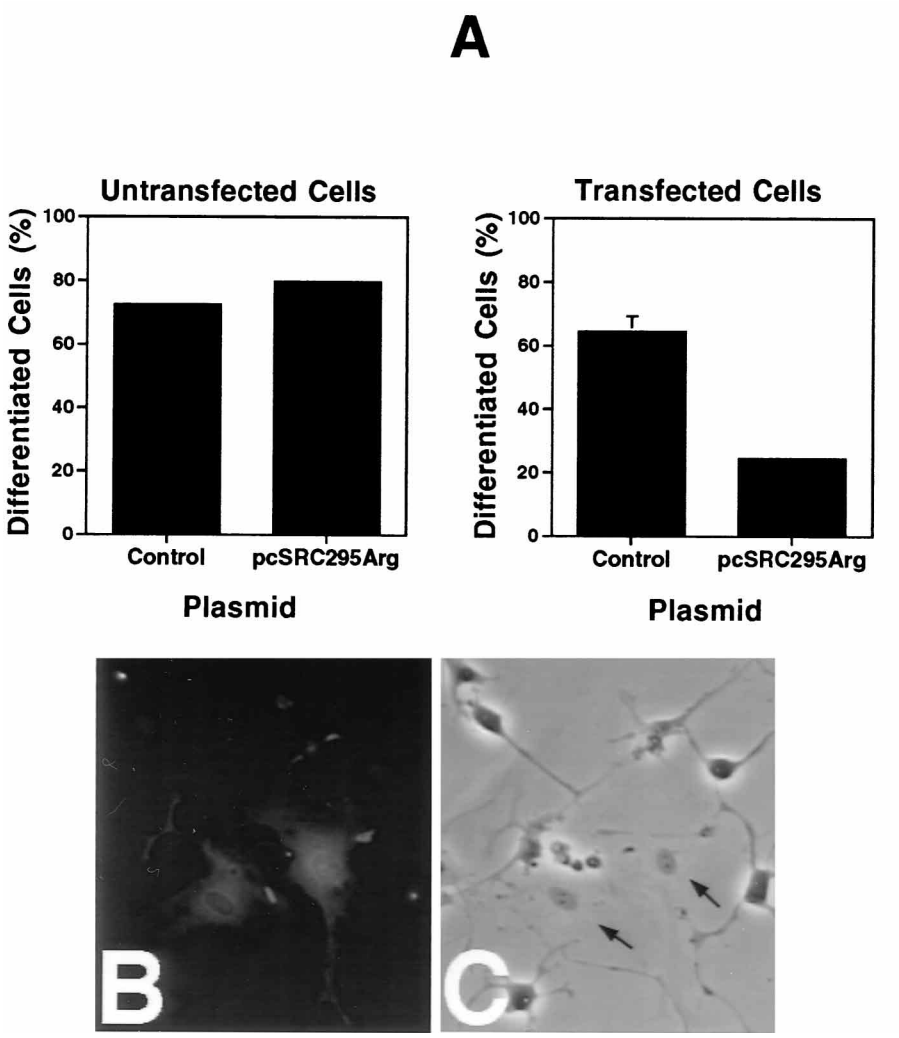

FIG. 5. Effect of dominant-negative Src (pcSrc295Arg) on H19-7 cell differentiation induced by bFGF. H19-7 cells were cotransfected with pCMV–β-gal and either the control (pEVX) or pcSrc295Arg vector. The cells were stimulated with bFGF under differentiation conditions for 36 to 48 h as described in Materials and Methods. The β-galactosidase-expressing cells were scored after characterizing the differentiation states as discussed in Materials and Methods. The percentage of differentiated cells was obtained by dividing the number of differentiated, β-galactosidase-expressing cells by the total number of β-galactosidase-expressing cells. (A) For each vector, cells not expressing β-galactosidase (untransfected cells) were scored in the same well as the β-galactosidase-expressing cells (transfected cells). The total numbers of β-galactosidase-expressing cells counted were 302 and 300 for the control and pcSrc295Arg vectors, respectively. The total numbers of untransfected cells counted were 635 and 527 for the control and pcSrc295Arg vectors, respectively. The results are the mean and range of data from two independent experiments. (B and C) Micrographs of the H19-7 cells from panel A which were cotransfected with pcSrc295Arg and pCMV–β-gal vectors and then treated with bFGF. The cells were probed with anti-β-galactosidase antibodies followed by anti-rabbit fluorescein isothiocyanate-conjugated IgGs. The β-galactosidase-positive cells were observed by immunofluorescence microscopy (B); the same field of the cells was visualized by phase-contrast microscopy, and the β-galactosidase-positive cells are indicated by arrows (C). Magnification, ×200.

were obtained when the dominant-negative N17-Ras or C4-Raf cDNAs were microinjected into H19-7 cells and analyzed for inhibition of differentiation (22). Taken together with the observation that Raf is activated by bFGF and is sufficient to induce differentiation, these results suggest that Ras and probably Raf are required for the differentiation of H19-7 neuronal cells by bFGF. Furthermore, these results suggest that there are downstream targets of bFGF-activated Ras or Raf that are distinct from MEK or MAP kinase (ERK1 and ERK2).

DISCUSSION

To distinguish between the signaling pathways that lead to differentiation as opposed to growth, it is important to define the initial signals that are required and/or sufficient for this process. To define the signaling pathways required for bFGF-induced differentiation, we have used a conditionally immortalized rat hippocampal neuronal progenitor cell line, H19-7. Previous studies have shown that activation of MEK alone is insufficient, and Raf requires a MEK-independent signaling pathway in H19-7 cells to promote neuronal differentiation (20). In the present study, our results indicate that induction of MAP kinase (ERK1 or ERK2) is not required for differentiation by bFGF. Instead, experiments suggest that at least two discrete signaling pathways are required, one involving Src and the other involving activation of Ras and probably Raf but not MEK (summarized in Fig. 11).

Our results provide an alternative model to a widely accepted paradigm that extended activation of MAP kinase is necessary for neuronal differentiation by tyrosine kinase recep-

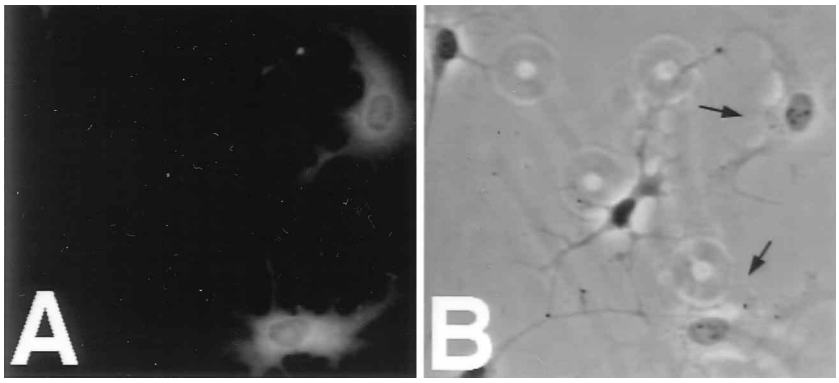

FIG. 6. Microinjection of anti-Src antibody (cst-1) blocked the differentiation of H19-7 cells induced by bFGF. H19-7 cells in DMEM–10% FBS were given microinjections of the affinity-purified cst-1 (4 μg/μl) and coinjections of rat IgG (1 μg/μl) as a marker for the injected cells. At 1 h after microinjection, the cells were switched to N2 medium, treated with bFGF, and shifted to 39°C for 36 to 48 h. Cells from the same field were visualized by immunofluorescence microscopy (A) and phase-contrast microscopy (B). Injected cells stained with fluorescein isothiocyanate-conjugated anti-rat antibodies are indicated by arrows. A total of 148 injected cells and 172 uninjected cells were analyzed. Magnification, ×200.

tors such as trk, the PDGF receptor, and the bFGF receptor (reviewed in reference 28). Although we cannot rule out the possibility that a low level of MAP kinase activity contributes to differentiation, our results with a synthetic inhibitor of MEK clearly demonstrate that induction of the ERKs is not required. These results, together with previous studies (20), indicate that activation of MAP kinase (ERK1 or ERK2) is neither necessary nor sufficient for differentiation by bFGF.

The observation that bFGF can differentiate cells without activation of MAP kinase is not surprising. First, there is precedent for bFGF signaling independent of MAP kinase activation. Inhibition of myoblast differentiation by bFGF has been shown to occur without significant activation of ERK1 or ERK2 (7). Second, bFGF can activate a number of different signaling pathways including phospholipase C, phosphatidylinositol 3-kinase, and Src, as well as other possible Ras-activated effectors (46). Since bFGF activates Raf in these cells and constitutively activated Raf is sufficient to differentiate H19-7 cells, one possibility is that Raf is necessary and sufficient for differentiation by bFGF. However, constitutive activation of Raf may transmit a different signal from bFGF-activated Raf, which is subject to other forms of regulation. For example, Raf-1 appears to be a potential substrate for its downstream effector MAP kinase (2). In addition, bFGF activates a number of other signaling pathways that may influence either directly or indirectly the signaling cascades activated by Raf. Since constitutively activated Raf requires activation of

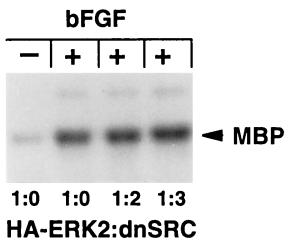

FIG. 7. Dominant-negative Src (pcSrc295Arg) did not block MAP kinase activation induced by bFGF. ΔRaf-1:ER cells were cotransfected with HA-ERK2 and the control (pEVX) or pcSrc295Arg vector. Increasing ratios, as indicated, of pcSrc295Arg to HA-ERK2 were transfected into ΔRaf-1:ER cells. After transfection, the cells were grown in DMEM–10% FBS overnight and then switched to N2 medium and shifted to 39°C for 36 h. Before being harvested, the cells were left untreated (−) or treated with bFGF (+) for 10 min. MAP kinase activity was assayed with MBP as a substrate as described in Materials and Methods. The reaction mixtures were resolved by SDS-PAGE, and the gel was dried and exposed to X-ray film for autoradiography. The [^{32}P]MBP bands are indicated by an arrow. These results are representative of two independent experiments.

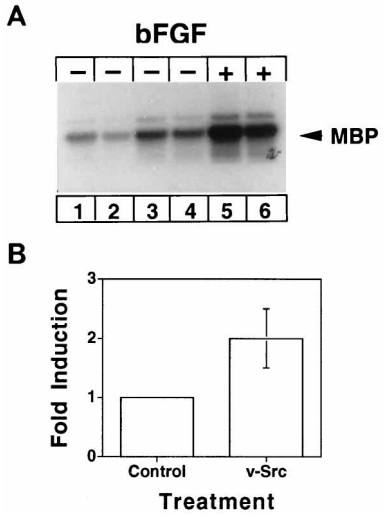

FIG. 8. v-Src is active in transfected H19-7 cells. (A) Activation of MAP kinase by v-Src at 33°C. The v-Src-expressing vector (2 μg) or the control vector (pEVX) (2 μg) along with HA-ERK2 expression vector (1 μg) were transfected into H19-7 cells as described in Materials and Methods. Before being harvested, the cells were left untreated (lanes 1 to 4) or treated with bFGF for 10 min (lanes 5 and 6). MAP kinase activity was assayed with MBP as a substrate as described in Materials and Methods. The reaction mixtures were resolved by SDS-PAGE, and the gel was dried and exposed to X-ray film for autoradiography. The [^{32}P]MBP bands are indicated by an arrow. Duplicate samples from independent wells are shown for the control vector (lanes 1 and 2) and v-Src vector (lanes 3 and 4). MAP kinase activity induced by bFGF from the cells transfected with the control vector (lane 5) or v-Src vector (lane 6) is also shown. The results are representative of two independent experiments. (B) v-Src induces the expression of p667 in transiently transfected H19-7 cells. The v-Src expression vector or the control vector (pEVX) was cotransfected with both pCMV–β-gal and p667, which is a mammalian expression vector containing the truncated Egr-1 promoter (pE425/+65) linked to CAT cDNA (34). At 24 h after transfection, the cells were switched to N2 medium at 39°C and treated or with bFGF or left untreated for 24 h before being harvested. CAT expression was assayed by ELISA, and the level of expression was normalized to the activity of β-galactosidase as a control for transfection efficiency. The fold induction is relative to the CAT expression from untreated cells transfected with the control vector (pEVX). These results are the mean and range of data from two independent experiments.

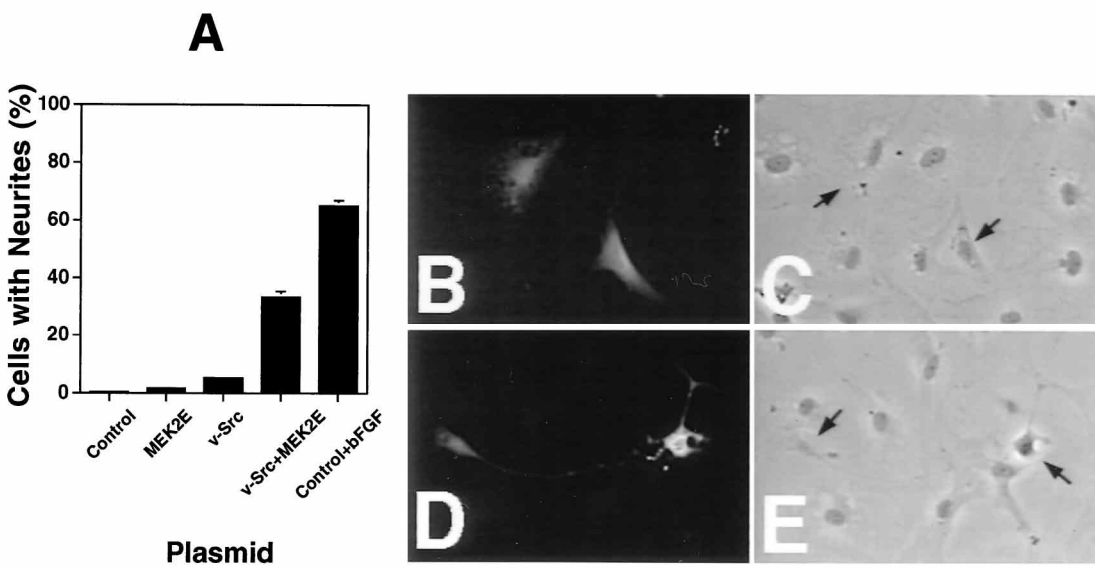

FIG. 9. Effect of vSrc on H19-7 cell differentiation. (A) H19-7 cells were cotransfected with pCMV–β-gal and various test vectors as indicated. The cells were treated with bFGF or left untreated under differentiation conditions for 36 to 48 h. β-Galactosidase-stained cells were characterized as described in Materials and Methods. The total numbers of β-galactosidase-expressing cells counted were 302, 391, 550, 270, and 367 for Control+bFGF, Control, MEK2E, vSrc, and vSrc+MEK2E, respectively. The results are the means and ranges of data from two independent experiments. (B and C) Micrographs of untreated H19-7 cells cotransfected with the control plasmid (pEVX) and pCMV–β-gal. Cells from the same field were visualized by immunofluorescence microscopy (B) and phase-contrast microscopy (C). (D and E) Micrographs of H19-7 cells cotransfected with v-Src, MEK2E, and pCMV–β-gal. Cells from the same field were visualized by immunofluorescence microscopy (D) and phase-contrast microscopy (E). Transfected cells stained with anti-β-galactosidase antibody were detected with secondary fluorescein isothiocyanate-conjugated antibodies and are indicated by arrows. Magnification, ×200.

MEK for differentiation whereas bFGF does not, our results indicate that bFGF uses a signaling pathway distinct from Raf.

Several lines of evidence point to Src family kinases as possible effectors of neurite outgrowth by bFGF receptor kinases. First, Src kinases have been reported to associate with the bFGF receptor (50) and are present at their highest levels in hematopoietic and neural cells (26). However, it should be noted that other investigators failed to detect a direct association between Src and the bFGF receptor, possibly suggesting a role for other Src family kinases (23). Second, Src family kinases are highly expressed in the growth cones of CNS neurons (3, 5, 27, 44). Third, neuronal differentiation is accompanied by extensive reorganization of the cytoskeleton to initiate the extension of neuritic processes, and many of the known substrates of Src such as $p130^{cas}$ (38) appear to be associated with cytoskeletal rearrangement. In addition, phosphorylated $p130^{cas}$ may serve as a signaling effector molecule for Src by binding to multiple cellular proteins. Fourth, neurite outgrowth stimulated by L1, N-cell adhesion molecule, and N-cadherin appears to occur via activation of the bFGF receptor tyrosine kinase (12). When cultures of cerebellar neurons from wild-type or Src-, Fyn-, or Yes-deficient mice were analyzed for neurite outgrowth on the neural cell adhesion molecule L1, the rate of neurite extension on L1 was reduced in Src-deficient but not in Fyn-deficient or Yes-deficient neurons (17). Finally, while development of the CNS proceeds normally in Src knockout mice (43), a likely explanation is that other Src family members can compensate for the defect. Consistent with this interpretation, Src activity is enhanced in neuronal cells from Fyn-deficient mice, which have abnormal hippocampal development, defective long-term potentiation, and impaired memory (15). In our cells, in vitro assays of Src kinase activity detected a discrete but modest activation. Although the studies with dominant-negative Src and the anti-Src antibody are consistent with a role for Src, it is possible that other Src family members alone or in combination also contribute to the bFGF differentiation process. Taken together, these observations demonstrate that Src kinases are an important effector of the bFGF receptor kinase and can act as a mediator of neurite outgrowth.

However, the results from our study suggest that Src alone is not sufficient for neurite outgrowth, and bFGF requires activation of another signaling pathway. While the combination of

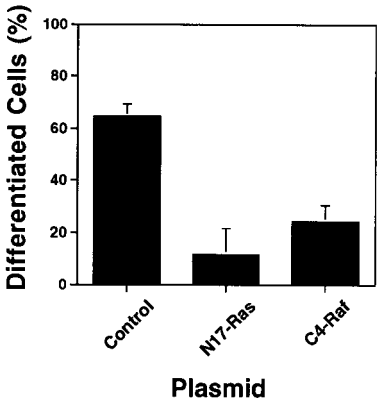

FIG. 10. Effect of N17-Ras and C4-Raf on H19-7 cell differentiation induced by bFGF. H19-7 cells were cotransfected with the control (pEVX) or dominant-negative Ras (N17-Ras) or Raf (C4-Raf), along with pCMV–β-gal vectors. The transfected cells were treated as described in Materials and Methods. The β-galactosidase-expressing cells were scored for differentiation as judged by morphological changes. The percentage of differentiated cells was obtained by dividing the number of differentiated, β-galactosidase-expressing cells by the total number of β-galactosidase-expressing cells. The total numbers of β-galactosidase-expressing cells counted were 302, 150, and 287 for the Control, N17-Ras, and C4-Raf vectors, respectively. The results are the means and ranges of data from two independent experiments.

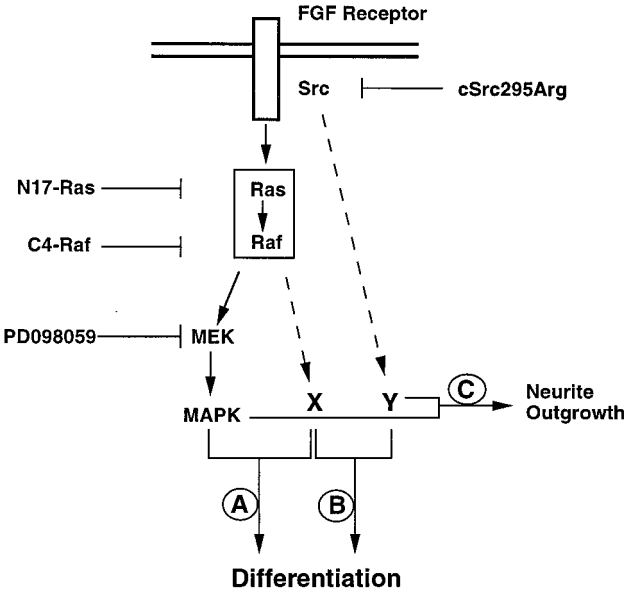

FIG. 11. Model of the signaling pathways involved in differentiation of the H19-7 hippocampal neuronal cells by bFGF. (A) Constitutive activation of Raf-1 kinase can differentiate H19-7 cells. In addition to the MAP kinase pathway, an alternative pathway designated X is required for Raf-1-induced differentiation (20). (B) bFGF can induce the differentiation of H19-7 cells; this differentiation requires Src as well as Ras and Raf. MAP kinase activation is not required for bFGF-induced differentiation; therefore, Ras and Raf probably activate at least one unknown factor that may correspond to X in pathway A. Src appears to activate a Ras- and Raf-independent pathway, designated Y in the figure. (C) Constitutive Src and MEK activation can promote neurite outgrowth but not the rounded and refractile cell bodies characteristic of differentiated H19-7 cells.

v-Src and constitutively activated MEK can mimic, at least in part, the signals required for neurite extension, activation of MEK does not appear to be required by bFGF. In contrast, the dominant-negative Ras blocks the transmission of signals by bFGF through the upstream activator SOS, implicating Ras in the differentiation process. Although Ras is required for signaling through Src in some systems (10, 37), there are several reports suggesting that Src can activate a distinct signaling cascade. First, mutation of Raf to remove the Ras binding site did not prevent activation of Raf by Src, implying that Src and Ras activate Raf-1 through different mechanisms (32). Second, transformation of NIH 3T3 cells by v-Src causes elevation of the level of cyclin E as well as of cyclin D1, whereas only the cyclin D1 level is elevated in v-H-ras and v-Raf transformants (25). These results suggest that Src induces cyclin E by a Ras-independent pathway. Barone and Courtneidge (4) recently showed that in NIH 3T3 cells, Myc, but not Fos or Jun, was able to rescue the inhibition of PDGF-stimulated DNA synthesis by dominant-negative Src whereas Fos and Jun, but not Myc, rescued the block conferred by dominant-negative Ras. Finally, coexpression of v-Src and v-Ras with baculovirus vectors results in the synergistic activation of MAP kinase (48). Similarly, in our studies, we also observed synergistic induction of neurite outgrowth with v-Src and activated MEK, a downstream effector of Ras. Furthermore, no activation of the Ras/Raf/MAP kinase pathway by v-Src was detected at the nonpermissive temperature in H19-7 cells (22). While there is clearly some cross talk between them under some conditions, Src and Ras activate two discrete signaling pathways that appear to play a critical role in both growing and differentiating systems.

Effectors of neuronal differentiation other than receptor tyrosine kinases may use different signaling pathways. Induction of PC12 cell differentiation by bone morphogenesis protein-2, activin A (18), or the GTPase-inactivating mutants Gα16 and Gαq (16) involves little or no activation of MAP kinases. When PC12 cells were differentiated with GTPase-inactivating mutants, sustained activation of Jun N-terminal kinases was detected and suggested to be responsible for the differentiation. From these results, it appears that more than one signaling pathway leads to differentiation of PC 12 cells.

Our results are consistent with some of the conclusions derived from studies of PC12 cells. The observation (45) that stimulation of the Ras/Raf/MAP kinase pathway by various PDGF receptor mutants is insufficient to sustain PC12 cell differentiation is consistent with our observation that the MAP kinase pathway is insufficient for H19-7 cell differentiation by bFGF (20). These authors further concluded that either Src or phospholipase C-gamma activation in addition to the Ras/Raf/MAP kinase pathway may be required for differentiation. Studies with dominant-negative mutants in PC12 cells implicate Src as well as Ras in this process (37). In H19-7 cells, inhibitors of protein kinase C, a downstream effector of phospholipase LC-gamma, do not block neuronal differentiation by bFGF (22). However, in both PC12 cells (37) and H19-7 cells (8), calcium, which is mobilized in response to phospholipase C activation, is able to activate Src. Thus, a requirement for activation of the Src signaling pathway could explain these observations. However, not all differentiating pathways in H19-7 cells and PC12 cells are identical. H19-7 cells are less responsive to constitutively activated MEK or v-Src and differ in their requirement for MAP kinase activation. Our results suggest that at least two discrete signaling pathways involving Src and Ras are required for bFGF-induced differentiation. It is interesting that PDGF-stimulated DNA synthesis in NIH 3T3 cells also requires distinct Ras- and Src-mediated signaling pathways (4). Elucidating the differences in the downstream targets of these pathways, such as the requirement for activation of MAP kinase, should enhance our understanding of how similar signals lead to distinct biological outcomes.

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