The Neurotrophic Activity of Fibroblast Growth Factor 1 (FGF1) Depends on Endogenous FGF1 Expression and Is Independent of the Mitogen-activated Protein Kinase Cascade Pathway*

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The expression of fibroblast growth factor (FGF) 1, a potent neurotrophic factor, increases during differentiation and remains high in adult neuronal tissues. To examine the importance of this expression on the neuronal phenotype, we have used PC12 cells, a model to study FGF-induced neuronal differentiation. After demonstrating that FGF1 and FGF2 are synthesized by PC12 cells, we investigated if FGF1 expression could be a key element in differentiation. Using the cell signaling pathway to determine the effects of FGF1 alone, FGF1 plus heparin, or a mutated FGF1, we showed an activation to the same extent of mitogen-activated protein (MAP) kinase cascade and MAP kinase (extracellular regulated kinase 1). However, only FGF1 plus heparin could promote PC12 cell differentiation. Thus, the MAP kinase pathway is insufficient to promote differentiation. Analysis of the PC12 cells after the addition of FGF1 plus heparin or FGF2 demonstrated a significant increase in the level of FGF1 expression with the same time course as the appearance of the neuritic extensions. Transfection experiments were performed to enhance constitutively or after dexamethasone induction the level of FGF1 expression. The degree of differentiation achieved by the cells correlated directly with the amount of FGF1 expressed. The MAP kinase pathway did not appear to be involved. Interestingly, a 5-fold increase in FGF1 in constitutive transfected cells extended dramatically their survival in serum-free medium, suggesting that the rise of FGF1 synthesis during neuronal differentiation is probably linked to their ability to survive in the adult. All of these data demonstrate that, in contrast to the MAP kinase cascade, FGF1 expression is sufficient to induce in PC12 cells both differentiation and survival. It also shows that auto- and trans-activation of FGF1 expression is involved in the differentiation process stimulated by exogenous FGFs through a new pathway which remains to be characterized.

FGF1 and 2 are widely distributed in the peripheral and central nervous systems in the adult. In rat brain, FGF2 is present in most neurons within the cerebral cortex (1), hippocampus (2), and cerebellum (3). High levels of FGF1 expression have been observed in motor neurons, primary sensory neurons, and retinal ganglion neurons (4, 5). In chick brain, the expression of FGF1 is developmentally regulated (6). In bovine and rat embryonic retina, all neuronal layers express FGF1 with an appearance corresponding to their sequential differentiation (7, 8). In rat, the level of FGF1 expression remains uniformly low throughout the embryonic period until postnatal day 7. Thereafter, it increases rapidly, reaching a maximum in the adult retina. In the intermediate central nervous system, subclasses of FGF receptors appear to be down-regulated during development (9), and during retinal embryonic development, the expression of FGFR1 and FGFR2 follows the retinal layering (10). These patterns of FGF expression suggest that these growth factors are involved in the integrity, development, and differentiation of the central nervous system. In fact, in vitro studies have shown that FGF1 promotes the survival of photoreceptors (11) and the neuritic outgrowth of dissociated retinal ganglion cells (12). FGF1 also inhibits pigmentation of immature pigment epithelium cells of embryonic chick retina and stimulates ganglion cell differentiation (13). FGF2 promotes the survival of neurons of the peripheral (14) and central nervous systems (15, 16) and delays photoreceptor degeneration in a retinal degeneration model (17, 18).

These FGF activities in vitro systems together with the temporal and spatial expression patterns of FGF in embryonic and adult neuronal tissues suggested that the FGF expressed by neuronal cells could be involved in the mediation of their neurotrophic activity. To investigate whether the expression of FGF1 by neuronal cells was implicated in the differentiation and neuronal survival, we have used PC12 cells as an in vitro model. This cell line was derived from a rat adrenal tumor (24) and responded to NGF and FGF by the extension of neurites and the acquisition of sympathetic neuronal phenotype. The transition of chromaffin phenotype to neuronal phenotype by NGF and FGF is accompanied by events mediated by the activation of high affinity tyrosine kinase receptors and the activation of the MAP kinase cascade. This is a main signaling pathway for cell proliferation, differentiation, and transformation and appears to mediate differentiation of PC12 cells induced by NGF and FGF2 (25–28).

In this study, we show that PC12 cells expressed both FGF1 and FGF2.
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FGF1 and FGF2. These cells were treated with different stimuli: FGF1-heparin, FGF2, and NGF (promoters of differentiation) or Lys-132-mutated FGF1 (FGF1-132E) and FGF1 alone (which do not promote differentiation). The expression of FGF1 at the transcript and protein level in parallel with the activation of the MAP kinase cascade was investigated in stimulated cells as a function of neuronal differentiation. The effect of FGF1 expression on neuronal differentiation was also examined in transfected cells in which FGF1 expression was under the control of a constitutive or dexamethasone-inducible promoter.

We show that the expression of FGF1 was activated only by exogenous FGF stimuli (FGF2 and FGF1-heparin) which are neurotrophic for PC12 cells and was unchanged when cells remained undifferentiated upon treatment with FGF1 alone or mutated FGF1-132E. In contrast, the MAP kinase cascade was similarly activated in stimulated cells whether or not they differentiated in sympathetic neurons. Accordingly, in transfected cells the expression of FGF1 strictly correlated with the differentiated phenotype and increased the survival of PC12 cells. We thus propose that the activation of the MAP kinase cascade is insufficient to induce the differentiation and survival of PC12 cells, and that the expression of FGF1 stimulated by exogenous FGF stimuli is a key element in the neurotrophic activities of the FGF.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant FGF1 was produced and purified in our laboratory (INSERM XPl18). FGF1-132E was from one of our laboratories (G. G.-G.). In the mutant FGF1, the lysine 132 was mutated to glutamic acid, and this mutation decreased the affinity of FGF1 to heparin (29). Human recombinant FGF2 was a kind gift from Farmitalia, Carlo Erba. The recombinant expression vector (pSVL-FGF1-134) was obtained from Dr. J. aye (Rhone-Poulenc-Rohrer) (30). The FGF-2-, 3- and 7-kDa fusion protein, was a gift from Drs. Lappi and Baird (Whitter Institute, San Diego).

Cell Cultures—Stock cultures of rat PC12 cells (originally obtained from P. Brachet, Angers) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 5% horse serum, and antibiotics at 37 °C in a humidified atmosphere of 5% CO2, air. Culture media were renewed every 3 days. PC12 cells transfected with pSVL-FGF1-134 were maintained as described for the control PC12 cells. PC12 cells transfected with pLk-FGF1-134 were maintained in DMEM supplemented with 10% fetal calf serum and 5% horse serum which were both depleted in glucocorticoid by fixation onto 1% agarose gels in DMEM supplemented with 10% fetal calf serum and 5% horse serum and antibiotics at 37 °C in a humidified atmosphere of 5% CO2, air. Culture media were renewed every 3 days. The morphology of the PC12 cells stimulated by exogenous factors and of the transfected cells was examined after 3–4 days of culture.

Cell Survival Assay—PC12 cells and constitutive transfected cells were washed three times with PBS, dissociated in PBS, 10 mM EDTA, and plated in a 24-multidish plate at a density of 5 × 104 cells/ml in serum-free medium. At the indicated times, trypan blue exclusion tests were performed.

RT-PCR Assay—RNA preparations and RT-PCR assay were performed as described previously by Renaud et al. (33). Total RNA was isolated from cultured cells using the guanidium isothiocyanate method (34). One μg of RNA and 170 pg of tobacco leaf nitrate reductase transcripts were reverse transcribed in 30 μl of 50 mM Tris-HCl, pH 8.9, 3 mM MgCl2, 75 mM KCl, 2.5 μM random hexanucleotide primers, 300 units of Maloney murine leukemia virus (Life Technologies, Inc.) and 1/10 of the reverse transcribes were amplified in 100 μl of 50 mM Tris-HCl, pH 8.9, 7 mM MgCl2, 50 mM KCl, 15 mM ammonium sulfate, 0.17 mg/ml bovine serum albumin, 1 μM of dNTP mix, 15 pmol of each specific primer with 1 unit of Taq polymerase (Eurobra) as described previously (33). In a preliminary experiment, the level of FGF1 or FGF2 in PC12 cells was tested by RT-PCR after 20–40 cycles of amplification, and we determined that the linear exponential FGF1/FGF2 amplification phase is comprised between 26 and 33 cycles (data not shown). However, some small RT-PCR efficiency differences between each experiment occurred. Thus, to ensure that the amplification was in the exponential phase, for each experiment aliquots were withdrawn at three different cycles and analyzed. For FGF1 and FGF2 amplification, the oligomers were chosen in different coding exons to avoid amplification of genomic DNA. Specific oligomers were used for rat (rFGF1S, rFGF1AS) and human (hFGF1S, hFGF1AS) FGF1 amplification: rFGF1AS (5'-AAG CCC GTC GGT GTC CAT GG-3') and rFGF1S (5'- GAT GCC ACA GTG GAT GGG AC-3') generated a 135-bp fragment; hFGF1AS (5'-TCC GAG GAC CCC TGC AG-3') and hFGF1S (5'-TCC GAC TTC CCA GTG AG-3') generated a 135-bp fragment. The two FGF1 oligomers (antisense primer 5'-CCC AGT TCG TTT CAC CAC C-3', sense primer 5'-CAT TAA AAG TGT GTT GTG CAA ACC-3') generated a 174-bp fragment, the two NGF oligomers (antisense primer 5'-CTC CAA CCC ACA CAC TGA CA-3', sense primer 5'-TCT GTC GCT GCC ACC CAC TG-3') generated a 342-bp fragment, and the nitrate reductase-specific oligomers (antisense primer 5'-GCT GAA TCC ATT GCA ATT TCC-3', sense primer 5'-AGG AGC TGA TGT GTT GCC CCG-3') generated a 75-bp fragment. The FGF1 or FGF2 or NGF and nitrate reductase-amplified products were electrophoresed on 10% polyacrylamide gel, blotted onto Hybond N+, and hybridized with specific FGF1, FGF2, NGF, and NR probes. X-OMat ARS x-ray film (Eastman Kodak Co.) was exposed for different periods, depending on the intensity of the signal.

FGF1 Expression by Analysis by Enzyme Immunonassay (EIA) and Western Blot—Native PC12 cells, FGF1-treated PC12 cells, and FGF1-transfected PC12 cells were lysed in PBS, pH 7.5, containing 2 mM NaCl, 0.1% Triton X-100. The protein concentration of the lysates was determined using a BCA kit (Pierce). The lysates were used to assay FGF1 by EIA and Western blot. Cell lysates (1 mg) were incubated with 100 μl of heparin-Sepharose (Pharmacia Biotech Inc.) in PBS, 0.6 mM NaCl. After one night of absorption at 4 °C, the heparin-Sepharose was washed twice with the binding buffer, and the heparin-binding proteins were eluted in PBS, 2 mM NaCl. The FGF1 was quantified using a second antibody said phase E1A as described previously by Oliver et al. (35). The cell lysates were in blotting analysis. A lane containing solely heterologous and constitutive transfected cells and from control PC12 cells were incubated with 30 μl of heparin-Sepharose in 0.6 mM NaCl as for EIA. Heparin-binding proteins were eluted in 30 μl of Laemmli buffer and electrophoresed on 18% SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes (Optitran BA-53, Schleicher & Schuell), the blots were incubated with 20% BSA, 1% intact proteins were blotted the day before. The blots were probed with rabbit polyclonal FGF1 antibody (diluted 1:500) in 150 mM NaCl, 100 mM sodium phosphate buffer, pH 7.6, containing 0.1% milk protein. The FGF1 antibody, directed against human recombinant FGF1, has been produced in our laboratory, and its specificity has been previously described (35). The filter was washed three times in the aforementioned buffer, incubated with biotinylated anti-rabbit IgG from donkey (diluted 1:400; Amersham Corp.), then with streptavidin-horse-radish peroxidase conjugate (diluted 1:400; Amersham Corp.), and finally developed with ECL Western blotting detection reagents (Amersham).
Expression of FGF1 and FGF2 Transcripts during the Differentiation of PC12 Cells—PC12 cells express a basal level of FGF1 and FGF2 mRNAs (Fig. 1A). When treated with FGF1 and heparin, PC12 cells extend neurites and increase their steady state levels of FGF1 (Fig. 1B) and FGF2 (Fig. 1D) mRNAs. The level of FGF1 transcripts remained constant during the first hours of treatment when no neuritic extensions are observed and increased progressively from day 1 to 5 with the same time course as the neuritic extension process (Fig. 1E). Short extensions appeared after 1 day of treatment and increased greatly in size from day 3 to 5. In contrast, the amount of FGF1 transcripts was similar in proliferating and confluent control cells cultured for 1, 3, and 5 days without FGF (Fig. 1C).

Neuritic Extensions in PC12 Cells Treated with FGF1, Mutated FGF1, FGF2, and NGF—Cells were treated in DMEM in the presence (Fig. 2) or absence of serum (data not shown), with FGF1 (100 ng/ml), FGF1K132E (5 μg/ml) in the presence or absence of heparin (10 μg/ml). The cells were fixed with 4% paraformaldehyde, incubated with anti-ERK1, 1/500, or nonimmune serum, 1/500, in 0.2% bovine serum albumin. Immunocytochemistry—Native PC12 cells and FGF1 constitutive transfected PC12 cells were plated onto poly-L-lysine-coated glass coverslips in 12-well plates at a density of 5 × 10⁵ cells/ml, treated for 3 days with FGF1 (100 ng/ml) in the presence or absence of heparin (10 μg/ml). The cells were fixed with 4% paraformaldehyde, incubated with anti-ERK1, 1/500, or nonimmune serum, 1/500, in 0.2% bovine serum albumin. Immunocytochemistry—Native PC12 cells and FGF1 constitutive transfected PC12 cells were plated onto poly-L-lysine-coated glass coverslips in 12-well plates at a density of 5 × 10⁵ cells/ml, treated for 3 days with FGF1 (100 ng/ml) in the presence or absence of heparin (10 μg/ml). The cells were fixed with 4% paraformaldehyde, incubated with anti-ERK1, 1/500, or nonimmune serum, 1/500, in 0.2% bovine serum albumin.

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Figure 2. Effects of FGF2, FGF1, and FGF1K132E on neuritic extension in PC12 cells. PC12 cells were plated onto poly-L-lysine-coated dishes in DMEM supplemented with 10% fetal calf serum and 5% horse serum. Twenty-four hours after plating, the cells were treated with 100 ng/ml FGF1 (2), 100 ng/ml FGF1 plus 10 μg/ml heparin (3), 10 ng/ml FGF2 (4), 50 ng/ml NGF (5), 5 μg/ml FGF1K132E (6), 5 μg/ml FGF1 (7), or 10 μg/ml heparin (8). Untreated cells were used as a control (1). The morphology of the cells was examined after 4 days of treatment (magnification ×75).

Figure 3. Analysis by RT-PCR of FGF1 transcripts in differentiated and undifferentiated PC12 cells. Total RNA was extracted from PC12 cells after 3 days of treatment with 100 ng/ml FGF1, in the absence (lane 2) or presence (lane 3) of heparin (10 μg/ml), with 10 ng/ml FGF2 (lane 4), with 100 ng/ml of NGF (lane 5) or with 100 ng/ml FGF1K132E plus heparin (10 μg/ml) (lane 6). Untreated cells cultured for 3 days were used as control (lane 1). One μg of each RNA preparation was assayed along with nitrate reductase transcripts to RT-PCR as in Fig. 1A. After 28 cycles, the amplification products were electrophoresed, analyzed by Southern blotting, and hybridized with FGF1 and nitrate reductase-specific probes. This experiment has been independently performed three times with similar results.

Figure 4. PC12 cells transfected with constitutive FGF1 expression vectors. PC12 cells were cotransfected with 10 μg of pSVL-FGF1-134 vector DNA and 1 μg of PSVL2-neo using Lipofectin reagent as described under “Experimental Procedures” and stable transfected lines were isolated after 15 days of selection in geneticin (0.5 mg/ml) containing medium. The established clones were cultured in serum containing DMEM, and their morphology was examined after 3 days of culture. An undifferentiated transfected clone (B7) (1 and 3) and a differentiated one (B12) (2 and 4) are shown at magnification ×70 (1 and 2) and ×180 (3 and 4).

Transfected clones, in particular transfected cells which had no neuritic extensions remained undifferentiated (data not shown). An undifferentiated (B7) and two fully differentiated
(B12 and B18) clones were selected for further analysis. It is worth noting that, during the selection process, few isolated cells or small colonies with highly differentiated phenotype were observed but could not be expanded and cloned.

Expression of FGF1 in Transfected Cells—To examine whether there was a correlation between FGF1 expression and the differentiation state of transfected cells, the expression of FGF1 was examined in the transfected clones at the mRNA and protein levels. After reverse transcription, human FGF1 mRNA was amplified with specific human FGF1 oligomers (hFGF1S and hFGF1AS). To control the specificity of the oligomers, RNA isolated from human mammary epithelial cell line MDA-MB-231 was used as positive control. The amplification products derived from transfected cells after 30 cycles, from PC12 cells (1) and MDA-MB-231 (2) after 40 cycles were analyzed by Southern blotting and hybridized with FGF1 and nitrate reductase-specific probes. Three independent RT-PCR were performed with similar results. B, analysis by RT-PCR of rat FGF1 transcripts. RNA from PC12 cells (1), MDA-MB-231 (2), differentiated B12, B18 (3, 4), and undifferentiated B7 (5)-transfected cells were assayed by RT-PCR using rFGF1S1S and rFGF1AS primers. Amplified products were analyzed as in A except that hybridization was performed with an internal rat specific FGF1 primer. C, analysis by EIA of FGF1 protein levels. 1 mg of protein lysate from native PC12 cells, from PC12 cells stimulated for 3 days with FGF1 (100 ng/ml) in presence or absence of heparin (10 μg/ml), from differentiated clones (B12, B18), and nondifferentiated clone (B7)-transfected PC12 cells was concentrated on heparin-Sepharose column affinity, and FGF1 present in the lysates was quantified by an EIA as described under “Experimental Procedures.” The results are expressed as nanograms of FGF1 per mg of total protein and are the mean value of three independent assays. D, undifferentiated transfected cells retained the capacity to extend neurites. Undifferentiated FGF1-transfected PC12 cells (clone B7) were cultured for 3 days in DMEM supplemented with 10% fetal calf serum and 5% horse serum without exogenous stimulus (1) or in presence of 100 ng/ml FGF1 plus 10 μg/ml heparin (2) (magnification × 115).

Fig. 5. Analysis of FGF1 expression in FGF1 transfected PC12 cells. A, analysis by RT-PCR of human FGF1 transcripts. 1 μg of total RNA from PC12 cells (1), differentiated B12, B18 (3 and 4) and undifferentiated B7 (5) transfected cells were assayed along with nitrate reductase transcripts by RT-PCR using hFGF1S1S and hFGF1AS primers, as described in Fig. 1A. RNA isolated from human mammary epithelial cell line MDA-MB-231 was used as positive control (2). The amplification products derived from transfected cells after 30 cycles, from PC12 cells (1) and MDA-MB-231 (2) after 40 cycles were analyzed by Southern blotting and hybridized with FGF1 and nitrate reductase-specific probes. Three independent RT-PCR were performed with similar results. B, analysis by RT-PCR of rat FGF1 transcripts. RNA from PC12 cells (1), MDA-MB-231 (2), differentiated B12, B18 (3, 4), and undifferentiated B7 (5)-transfected cells were assayed by RT-PCR using rFGF1S1S and rFGF1AS primers. Amplified products were analyzed as in A except that hybridization was performed with an internal rat specific FGF1 primer. C, analysis by EIA of FGF1 protein levels. 1 mg of protein lysate from native PC12 cells, from PC12 cells stimulated for 3 days with FGF1 (100 ng/ml) in presence or absence of heparin (10 μg/ml), from differentiated clones (B12, B18), and nondifferentiated clone (B7)-transfected PC12 cells was concentrated on heparin-Sepharose column affinity, and FGF1 present in the lysates was quantified by an EIA as described under “Experimental Procedures.” The results are expressed as nanograms of FGF1 per mg of total protein and are the mean value of three independent assays. D, undifferentiated transfected cells retained the capacity to extend neurites. Undifferentiated FGF1-transfected PC12 cells (clone B7) were cultured for 3 days in DMEM supplemented with 10% fetal calf serum and 5% horse serum without exogenous stimulus (1) or in presence of 100 ng/ml FGF1 plus 10 μg/ml heparin (2) (magnification × 115).
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Fig. 6. Analysis of PC12 cells transfected with inducible FGF1 expression vectors. A, 150 μg of protein lysates isolated from PC12 cells (1 and 2), from inducible FGF1-transfected cells nondifferentiated D1 clone (3 and 4) and differentiated D49 clone (5 and 6), and from constitutive FGF1-transfected cells nondifferentiated B7 clone (7) and differentiated B12 clone (8) were concentrated on heparin-Sepharose and the level of FGF1 produced by these cells was examined by Western blot as described under “Experimental Procedures.” PC12 cells and inducible transfected cells (D1 and D49) were maintained in glucocorticoid depleted medium (1, 3, and 5) or treated with dexamethasone (5 × 10⁻⁷ M) for 3 days (2, 4, and 6). Constitutive transfected cells (B7 and B12) were cultured in DMEM for the same time. Under these conditions, only the inducible transfected clone D49 in presence of dexamethasone (6) and the constitutive B12 transfected clone (8) extended neurites. B, the morphology of the D49 cells nontreated (1) or treated with dexamethasone (2) for 3 days was presented. C, in the differentiated inducible transfected cells D49, the level of FGF1 was examined after 1, 3, and 5 days of dexamethasone treatment (respectively lanes 2, 3, and 4). Nontreated cells were presented in lane 1. Fifty μg of protein lysates were concentrated on heparin-Sepharose and FGF1 detected by Western blot as in A.

The neurotrophic process, PC12 cells were transfected with the dexamethasone-inducible expression vector pLK-FGF1-134. Different stable clones were isolated. In response to dexamethasone, some clones (as the D49 clone) extended neurites (Fig. 6B), whereas other clones maintained an undifferentiated phenotype (as the D1 clone). FGF1 synthesis in these clones was analyzed by Western blot (Fig. 6, A and C). As expected, only the D49 clone, induced by dexamethasone to differentiate, depicted an increased level of FGF1 to the same extent as constitutive transfected cells, B12 (Fig. 6A). This increase appeared 1 day after treatment and before the appearance of the extension of neurites which followed the same time course as in FGF1-treated cells (Fig. 6C). Dexamethasone, by itself, had no effect on the process of differentiation promoted by exogenous FGF1 (data not shown) and had no influence on FGF1 expression in control PC12 cells (Fig. 6A).

Characterization of the Differentiation State of the Constitutive Transfected Cells—NGF stimulates the activity of the ChAT in PC12 cells (41), and therefore was evaluated in FGF1-treated cells in the presence or absence of heparin and in differentiated FGF1-transfected cells (B18) (Fig. 7A). As expected, FGF1 alone did not stimulate the ChAT activity, while in differentiated cells treated with FGF1 and heparin or in differentiated transfected cells, the ChAT activity was increased. The acetylcholine esterase activity which is stimulated in PC12 cells by FGF or NGF (42) also was evaluated in the two differentiated transfected clones (B12 and B18). The activity was increased four to eight times in differentiated transfected cells (Fig. 7B). The expression of the cell surface glycoprotein Thy-1, which is induced in PC12 cells differentiated after FGF2 or NGF treatment (43, 44), was also used as a marker of cell differentiation. The undifferentiated clone B7 and the PC12 cells expressed the same level of Thy-1 mRNA, while the differ-
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Cell survival analysis—The ability of FGF1 to promote the survival of constitutive transfected cells was also examined (Fig. 8). The survival of control PC12 cells and of undifferentiated (B7) and differentiated (B12) transfected cells was compared. After 4 h of culture in the absence of serum, the percentage of cell attachment was identical for the three clones. Within 24 h, 40% mortality was observed in all cases. After 3 days of culture in the absence of serum, 65% mortality for the control PC12 cells or the undifferentiated transfected cells (B7) was observed, while the viability of the differentiated transfected cells (B12) was unaffected. For the two undifferentiated clones, the percentage of mortality increased at day 4 to 80% and to more than 90% by day 7. The differentiated transfected cells remained almost 60% viable at day 4 and 40% at day 7. These cells could be maintained in the absence of serum for 1 month, and proliferation was resumed in culture by the addition of serum to the cultures.

The Differentiating Action of FGF1 on PC12 Cells Is Independent of the Transient Activation of MAP Kinase—To determine whether the neurotrophic activity of FGF1 in PC12 cells was mediated by the MAP kinase signaling pathway, the activation of the MAP kinases in cells treated by exogenous stimuli inducing or not cell differentiation and in the FGF1 constitutive transfected cells was examined. The activity of ERK1, immunoprecipitated from the cell lysates, was measured by phosphorylation of myelin basic protein. The stimulation of ERK1 after treatment of PC12 cells with NGF was used as a positive control (100% activation of ERK1).

Exogenous FGF1 induced the activation of ERK1 to the same extent as did NGF and FGF2 whether or not cells were induced to differentiate, that is in presence or absence of heparin (Fig. 9A). Similarly, cells treated with the mutated FGF*K132E and heparin, which remained undifferentiated, induced the same extent of ERK1 activity as differentiated cells treated with FGF2 or FGF1 and heparin.

The kinetic pattern of ERK1 activation (Fig. 9B) was studied after stimulation with NGF, FGF1, and FGF1*K132E in the presence or absence of heparin. FGF1 and the mutated FGF1*K132E stimulated ERK1 activity with a different pattern from the NGF one. After 5 min of stimulation the peak of activity was identical in NGF-, FGF1-, and FGF1*K132E-treated cells, but after 15 min, in FGF1-treated cells, ERK1 activity returned to basal level, while in NGF-treated cells, it remained elevated. Heparin, which was essential for differentiation, did not change the profile of ERK1 activation by FGF1 (Fig. 9B).

It has been shown previously that NGF, in contrast to epidermal growth factor which is a mitogenic factor for the PC12 cells, induced nuclear translocation of ERK1 (27). Therefore, we examined the subcellular localization of ERK1 in PC12 cells stimulated by FGF1 in the presence or the absence of heparin (Fig. 9C). The immunohistochemical analysis showed that, in both conditions, FGF1 was unable to induce nuclear translocation, while after 3 days of treatment with FGF1-heparin cells extended neurites but remained undifferentiated in presence of FGF1 alone.

While ERK1 has been shown to be involved in the neurotrophic activity of NGF, considering the diversity of MAP kinases, it was plausible that another MAP kinase could be implicated in the differentiating action of FGF1. Therefore we analyzed the activity of the upstream regulator, MAPKK. As for ERK1 activity, we showed that in undifferentiated cells treated with FGF1 alone, the MAPKK is activated to the same extent as in differentiated cells treated with FGF1 and heparin or with NGF (Fig. 10A).

In summary, it appears that the activation of the MAP kinases by FGF1 is independent of the differentiation state of the cells, and that, in contrast to NGF, FGF1 does not sustain ERK1 activity described as a key element of PC12 cell differentiation.

In the absence of exogenous stimuli, the transfected undifferentiated (B7) or differentiated (B12 and B18) cells exhibited a low basal ERK1 and MAPKK activities (Fig. 10A). This absence of activity was not due to an inhibition of ERK1 or MAPKK expression, since Western blot analysis showed that both were expressed at the same level in the transfected cells and in PC12 cells (Fig. 10B). Modification of the localization of ERK1 did not occur, as immunohistochemical analysis showed that ERK1 was localized mostly in the cytoplasm in both the transfected clones and PC12 cells (Fig. 10C). ERK1 could also be activated by treating the transfected clones by exogenous NGF for 5 min. In differentiated PC12 cells (treated with NGF for 3 days and then NGF and serum-depleted medium for one night) and in differentiated transfected cells (B18), 5 min of NGF stimulation activated ERK1 to the same extent (Fig. 11). The presence of potentially active FGF receptor in differentiated transfected clones was examined using FGF-saporin, a cytotoxic complex which enters the cells via the high affinity FGF receptors (45) (Fig. 12). Clones B7 and B12 and the PC12 cells were treated with 1 nM FGF-saporin, and after 3 days of treatment 47% of PC12 cells, 60% of B7 cells, and 43% of B12 cells were killed, confirming the presence of functional FGF receptors in the transfected clones (Fig. 12). These results demonstrate that the absence of differentiation observed under the various conditions described above could not be attributed to a default in FGF-R activation.

The Differentiating Action of FGF1 on PC12 Cells Is Independent of NGF Expression—To determine whether the neurotrophic activity of FGF1 on PC12 cells was mediated indirectly by NGF, the expression of this factor by cells treated with exogenous stimuli and in FGF1-constitutive or -inducible transfected cells was examined by RT-PCR. As shown (Fig. 13A), PC12 cells treated by exogenous FGFs (FGF1 or FGF1*K132E plus heparin and FGF2) expressed NGF mRNAs to the same extent as control PC12 cells. In constitutive FGF1-
transfected cells (Fig. 13B), endogenous FGF1 expression had no effect on NGF expression. In inducible FGF1-transfected cells, a decrease of NGF mRNA was observed after dexamethasone treatment in differentiated cells (clone D49) and in control transfected cell (clone D1). There was no overexpression of FGF1 nor extension of neurites in the D1 clone, suggesting that the down-regulation of NGF expression was independent of these properties and was due only to dexamethasone treatment. These data showed that exogenous FGFs, as well as endogenous FGF1 expression, did not regulate NGF expression in PC12 cells. Interestingly, we also observed that, in these cells, exogenous NGF induced its own expression (Fig. 13A). Thus, autoactivation of endogenous expression by exogenous neurotrophic factor may be a general phenomenon.

**DISCUSSION**

PC12 cells have been widely used as a model system for examining the molecular mechanisms by which FGF2 and NGF induce neuronal differentiation. In the present study, instead of comparing the effects of different neurotrophic (NGF/FGF) versus mitogenic (epidermal growth factor) factors, we have taken advantage of the heparin requirement in the neurotrophic process (43, 46) mediated by FGF1, to distinguish the specific responses implicated in the neurotrophic activity of this growth factor.

We show that FGF1 alone or the mutated FGF1 K132E, known to have decreased affinity for heparin (29, 40), does not induce neuronal differentiation, in contrast to FGF1 plus heparin. This indicates that FGF1 and FGF1-heparin may have distinct cellular targets. This is supported by data showing that FGF2 internalized by heparan sulfate proteoglycan or by the complex heparan sulfate proteoglycan/FGF-receptor is not targeted to the same intracellular compartments (47). In PC12 cells, the different intracellular fates of FGF1 and FGF1-heparin may determine its neurotrophic action.

A variety of cellular mechanisms are presumably involved in the process of differentiation induced by neurotrophic factors. The most important signaling pathway thought to be implicated in PC12 cell differentiation, promoted by NGF and FGF2, is the Ras/MAP kinase signaling system (26, 28, 48, 49). It has been argued that it is the duration of MAP kinase activation (25) and the nuclear translocation of ERK1 (26) induced by NGF and FGF2, but not by epidermal growth factor, which plays a key role in the generation of the neurotrophic action. In the present study, we show that FGF1, FGF1 K132E, and FGF1-heparin, which have distinct effects on PC12 cell differentiation, have the same profile of activation of ERK1 and do not alter the ERK1 subcellular localization. This suggests that the activation of the MAP kinases alone is not sufficient to induce differentiation and that FGF1 neurotrophic activity could be either mediated by a different cascade or could diverge from the MAP kinase signal beyond ERK1. In both cases an additional element must be involved.
Such an element could be the increased expression of FGF1 which, in contrast to the activation of the MAP kinase cascade, correlates strictly with the differentiation of PC12 cells, either treated with exogenous FGF or transfected with FGF1 expression vectors. In cells treated with exogenous FGF2 or FGF1 and heparin, kinetic analysis of FGF transcripts shows an increased amount of FGF mRNAs which had already occurred at day 1, before any morphological modifications of PC12 cells. This increase then followed the same time course as the neuritic extension process. In contrast, the level of FGF1 remained unchanged during the differentiation of NGF-treated cells. A similar situation was observed in expression of NGF mRNA during the differentiation of FGF-treated cells, suggesting different signaling pathways for FGF and NGF neurotrophic activities.

To confirm that the FGF increase, produced by PC12 cells stimulated by exogenous FGF, was sufficient to promote neuronal differentiation, constitutive and inducible FGF1-transfected cells were established. In PC12 cells treated with exogenous NGF or FGF, differentiation occurs after several days when cell proliferation ceases. Thus, stable transfection with a putative neurotrophic factor under the control of a constitutive expression promoter was performed assuming that the proliferation phase which precedes the differentiation process is enough to isolate colonies. As expected, several stable transfected clones which presented a morphological differentiated phenotype and an increase in ChAT and AChE activities and of Thy-1 mRNA were isolated. The proliferation rate of these differentiated clones and of PC12 cells were identical, but as expected, differentiated cells ceased to proliferate earlier than the undifferentiated cells (data not shown).
any exogenous neurotrophic stimuli, FGF1 constitutive transfected cells or transfected cells under the control of the murine mammary tumor virus-inducible promoter, expressed a differentiated phenotype with neuritic extensions only when the concentration of FGF1 reached a certain level of expression. Some constitutive transfected cells expressing an intermediate level of FGF1 displayed an undifferentiated or an incompletely differentiated phenotype (data not shown). In inducible transfected clones the extension of neurites followed the same time course of that in exogenous treated cells and in both cases the increase in FGF1 preceded the morphological changes. All of these data imply that FGF1 expression either stimulated by exogenous FGF or under the control of strong promoters in transfected cells is responsible for the neurotrophic activity.

In fact, increased expression of FGF1, in constitutive transfected PC12 cells, not only induces differentiation but also promotes the long term survival of differentiated cells in a serum-free medium, while control PC12 cells die rapidly and exhibit the characteristic pattern of DNA fragmentation associated with apoptosis (50). As exogenous FGF are known to increase survival of neuronal cells such as photoreceptors (11, 17, 18), cholinergic neurons, and retinal ganglion cells (51), in view of our data, the survival activity of exogenous FGF should depend on an increase in the expression of FGF1 or FGF2 in these neuronal cells.

Previous data have already demonstrated that the expression of FGF induced by the exogenous forms (autoactivation and transactivation of FGF) could be involved in the mediation of FGF biological activities. These forms of expression would stimulate cell proliferation in astrocytes and hippocampal neuronal cells and myogenic differentiation (21, 22). In lens epithelial cells, the increased expression of FGF1 prevents apoptosis, while the addition of FGF1 antisense oligonucleotides provokes cell death (33).

Our data also underline that the neuronal differentiation and survival activities of FGF1 could thus be controlled by a precise quantitative regulation of its level of expression. The importance of quantitative aspects of FGF has also been described for exogenous FGF. Guillemot and Cepko (13) demonstrated that the choice of fate of the bipotential neuroepithelium depends on the concentration of exogenous FGF. A 2–10 times difference in FGF2 concentration also controls the choice of ventral type mesoderm or notochord differentiation (52), and the proliferative, migratory, or differentiation responses of rat lens epithelial cells in vitro is dependent on the dose of FGF2 to which they are exposed (53).

The observation that an increase in FGF1 promotes neuronal differentiation and increases survival of PC12 cells suggests that in vivo the expression of FGF1 which reaches a maximum in adult neuronal tissues could be a key control step in the induction or inhibition of differentiation as well as the survival of nervous tissues. This may explain the need for a precise regulation of FGF1 gene expression which involves multiple promoters (54) as well as some regulatory elements located in the long and complex 5′- and 3′-untranslated regions of FGF1 transcripts (55–57). Studies demonstrating that endogenous FGF are localized in the nuclei of several types of cultured cells and tissues (20, 35, 58, 59) and that exogenous FGF can translocate to the nuclei at certain phases of the cell cycle (19, 60) suggest that FGF could act directly in the cell (23) possibly as nuclear transcription factors. This hypothesis is supported by cell-free experiments in which FGF2 was shown to modulate the transcription of Pkg-1 and Pkg-2 genes (61). In PC12 cells, FGF could be involved in the activation of genes necessary for neuronal differentiation and survival. In this context, the results of ongoing experiments to establish the subcellular localization of FGF1 in FGF1-treated cells and transfected PC12 cells as a function of differentiation would be of interest.

Neuronal cell death occurs naturally during development and is also a consequence of insult, aging, and degenerative disorders. Increasing tissue FGF levels might thus be beneficial in certain chronic and progressive neurodegenerative disorders.

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Addendum—While this paper was submitted, an analysis of the PC12 differentiation pathway demonstrated, in accordance with our results, that MAP kinase activation is insufficient for growth factor receptor-mediated PC12 differentiation (62).

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