Fibroblast Growth Factor Receptors Participate in the Control of Mitogen-activated Protein Kinase Activity during Nerve Growth Factor-induced Neuronal Differentiation of PC12 Cells*

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The current paradigm for the role of nerve growth factor (NGF) or FGF-2 in the differentiation of neuronal cells implies their binding to specific receptors and activation of kinase cascades leading to the expression of differentiation specific genes. We examined herein the hypothesis that FGF receptors (FGFRs) are involved in NGF-induced neuritogenesis of pheochromocytoma-derived PC1 cells. We demonstrate that in PC1 cells, FGFR expression and activity are modulated upon NGF treatment and that a dominant negative FGFR-2 reduces NGF-induced neuritogenesis. Moreover, FGF-2 expression is modulated by NGF, and FGF-2 is detected at the cell surface. Oligonucleotides that specifically inhibit FGF-2 binding to its receptors are able to significantly reduce NGF-induced neurite outgrowth. Finally, the duration of mitogen-activated protein kinase (MAPK) activity upon FGF or NGF stimulation is shortened in FGFR-2 dominant negative cells through inactivation of signaling from the receptor to the Ras/MAPK pathway. In conclusion, these results demonstrate that FGFR activation is involved in neuritogenesis induced by NGF where it contributes to a sustained MAPK activity in response to NGF.

Growth factors participate in axon growth, neuron survival in the nervous system during embryonic development, and in regeneration of peripheral nerves of vertebrate organisms (for reviews see Refs. 1–3). Several studies depicted the primordial role of NGF, FGF-1, and FGF-2 in the differentiation and survival of neuronal cells in vivo and ex vivo (4–6). Other studies suggested that the NGF/NGFR and the FGF/FGFR transduction pathways are interdependent. For example, in the early stages of embryonic chicken development, FGF mRNAs are expressed, and the decline of their presence is accompanied by NGFR mRNA expression and, ultimately, by a new round of de novo FGFR transcription (7). Moreover, FGF-2 stimulates NGFR gene promoter activity in a human neuroblastoma-derived cell line (8) and acts in synergy with NGF in neuronal stem cell differentiation and proliferation (9). Taken together, these observations imply that in the nervous system NGF and FGFs intervene alternatively and sequentially in neuronal differentiation and are, to some extent, interdependent and co-regulated.

The PC12 rat adrenal pheochromocytoma-derived cell line differentiates either into sympathetic neuron-like cells or into chromaffin-like cells (10). NGF, FGF-1, or FGF-2 differentiate PC12 cells into cells morphologically and biochemically resembling sympathetic neurons (11, 12). NGF signal transduction is mediated through the activation of tyrosine kinase cell surface receptors (13). The NGF transduction pathways proceed through p21ras (14) and B-Raf (15) activation, leading to the activation of MAPK kinase (MEK), which is sufficient for PC12 differentiation (16). The activation of the MAPK by NGF is insufficient, however, to mediate differentiation of PC12 cells (17), suggesting that other transduction pathways involving Shc, phospholipase C-γ, or yet unidentified MAPK kinase-dependent pathways (18, 19, 16) may intervene. FGFRs also signal through the activation of tyrosine kinase cell surface receptors. Four different structurally related FGF receptor sub-families (FGFR-1 to FGFR-4) have been identified, but little is known about the intracellular downstream signaling. However, it seems to involve Grb2/Sos and the MAPK pathway (20), phospholipase C-γ does not play a significant role in PC12 cell differentiation because FGF-2 stimulates neuronal differentiation in cells that express a mutated FGFR-1 that does not bind phospholipase C-γ (21, 23). Recently, a 90-kDa tyrosine-phosphorylated protein (named SNT, SLP, or R-SFR-2) was involved in FGF signaling (24–27) by participating in the sustained activation of the MAPK pathway through Grb2 and SHP-2 (27, 28).

In this study, we show that FGFR-2 is involved in NGF signaling leading to PC12 neuronal differentiation. We report that FGFRs expression and activity are modulated by NGF. Furthermore, we show that endogenous FGF-2 expression is induced by NGF and participates in FGFR activation. Finally, FGFR substrate; SLP, suc-1-associated NGF receptor target like protein; MBP, myelin basic protein; DN, dominant negative.
we demonstrate that the NGF-induced transduction pathways involving FGFRs depend upon SLP/FRS activation and sustained MAPK activity. The hypothesis that in the nervous system, FGFR and FGFR-2 are part of NGF signaling is discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Electroporation**—PC12 cells (ATCC CRL-1721) were grown on gelatin-coated Petri dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 5% horse serum, and 4.5 g/liter glucose at 37 °C in a 5% CO2 atmosphere. NGF or FGF-2 treatments were performed once at the beginning of the experiment. The plasmid RK5 containing a 1.3-kb insert encoding a tyrosine kinase activity-deficient human FGF receptor-2 exon IIIe (DNFGRF-2 (29)) was kindly provided by Dr. J. Schlessinger. PC12 cells (8 × 105) were electroporated with pRK5 (0.9 μg) and PSmveo (0.1 μg) at 170 V and 1800 microfarads. Isolated G418-resistant clones were tested for the presence of high affinity FGF-2 binding sites as described by Moscatelli (30) and by cross-linking to 125I-FGF-2.

**Neurite Outgrowth Assay**—Cells seeded at 2 × 105 cells/cm2 were tested for neurite outgrowth for up to 10 days under NGF (30 ng/ml) or FGF-2 (10 ng/ml) treatment. Cells with one or more neurites of a length at least twice superior to the cell diameter were scored as positive. Three independent experiments (100 cells/dish) were scored for each time point.

Neurite outgrowth studies were also performed in the presence of heparin (100 μg/ml) or neutralizing anti-FGF-2 antibodies (200 μg/ml; AB-33-NA; R&D System, Minneapolis, MN) or nucleotide-resistant high affinity RNA ligands to FGF-2. The 2′-amino-2′-deoxyxyrimeidine-containing RNA (31) was further stabilized against nucleases by substituting 9 rib mixins with 2′-deoxy-2′-O-methylurines and adding phosphorothioate caps to the 5′ and 3′ ends. These changes were incorporated in ligand NX-286: (5′-G GUGU/GAGGAAGCAGCGCGUGGQUUC (3′-P), where (5′-P) and (3′-P) represent the phosphorothioate caps (5′-d(TsTsTsTsTs) and d(TsTsTsTsTs-3′)), with “s” representing the phosphorothioate internucleoside linkage), 2′-amino-2′-deoxyxyrimidine nucleotides are italicized, and 2′-deoxy-2′-O-methylurine nucleotides are underlined. NX-286 binds to FGF-2 with a Kd of 50 nM. The sequence-scrambled analogue of this ligand, scNX-286, (5′-P) AGUGUGGGACGGUGQUUGCGGUGGUGCA (3′-P), or vascular endothelial growth factor ligand, NX-213 (32), binds to FGF-2 with about 10-fold lower affinity and was used as a control. The different FGF-2 binding molecules were added 10 min before NGF or FGF-2 treatment at 100–500 nm.

**125I-FGF Binding, Cross-linking, and Scatchard Analysis**—Human recombinant FGF-2 (Synergen, Boulder, CO; 10 μg) was iodinated with 1 mCi of 125I (ICN, Costa Mesa, CA) by the Iodo-Gen method to a specific activity of about 1.85 mCi/mg of protein. The iodinated RNA was further stabilized against nucleases by substituting 9 rib mixins with 2′-deoxy-2′-O-methylurines and adding phosphorothioate caps to the 5′ and 3′ ends. The sequence-scrambled analogue of this ligand, scNX-286, (5′-P) AGUGUGGGACGGUGQUUGCGGUGGUGCA (3′-P), or vascular endothelial growth factor ligand, NX-213 (32), binds to FGF-2 with about 10-fold lower affinity and was used as a control. The different FGF-2 binding molecules were added 10 min before NGF or FGF-2 treatment at 100–500 nm.

**RESULTS**

**NGF Modulates FGF-2 Binding on PC12 Cell Surface**—Previous studies have shown that FGFRs are present before the initiation of differentiation of PC12 cells (31). To monitor the evolution of the FGFRs in the early stages of NGF-induced differentiation, we carried out binding experiments with 125I-FGF-2 on PC12 cells treated for 0, 2, 6, 12, 24, 48, and 96 h with NGF. No competition between FGF-2 and NGF was observed (data not shown). After 2 h of NGF treatment, the level of FGF-2 binding to low affinity binding sites (proteo-heparan sulfates) increased by 7-fold and returned to the initial level after 24 h (Fig. 1A) as described previously (36). Binding of FGF-2 to its high affinity sites after NGF treatment resulted in a 3–4-fold increase in binding detected after 6 h, maybe because of a cooperation with low affinity binding sites and at 48 h (Fig. 1A). No change in FGF-2 binding was observed when cells were treated with EGF (not shown). To characterize the membrane-associated molecules that bind FGF-2, cross-linking experiments were carried out during NGF treatment as indicated above. Fig. 1B depicts a ~157-kDa cross-linked complex, similar to the binding experiments, the abundance of this complex was highest at 6 and 48 h. Cross-linking of 125I-FGF-2 was competed by a 100-fold excess of unlabeled FGF-2 (not shown). Additional bands of lower molecular weight were detected during NGF treatment (121 kDa more so at 48 h; 103 kDa at 2, 6, 12, and 48 h and, 83 kDa at 2 and 6 h; Fig. 1B). The number and dissociation constants (Kd) of 125I-FGF-2 binding to FGF receptors were determined by Scatchard analysis (33) in either untreated or 48-h NGF-treated cells. Two classes of receptor binding sites (class 1: Kd = 134 ± 27 pm, number of sites = 1.6 ± 0.7 fmol/106 cells; class 2: Kd = 957 ± 125 pm, number of sites = 5.12 ± 1.1 fmol/106 cells) were detected in untreated PC12 cells. After NGF treatment (48 h), the following binding values were observed: class 1, Kd = 160 ± 11 pm, number of sites = 3.6 ± 0.2 fmol/106 cells; class 2: Kd = 457 ± 55 pm, number of sites = 77 ± 2 fmol/106 cells (Fig. 1C).

**NGF Stimulates FGF-1 and FGF-2 Tyrosine Phosphorylation**—125I-FGF-2 cross-linked material from untreated or NGF-treated cells was immunoprecipitated with anti-FGFR antibodies. FGF-1 (~160 kDa) and FGF-2 (~157 kDa) were principally present in PC12 cells under NGF treatment at 48 h and 6 h, respectively (Fig. 2A). Northern blotting experiments with either one of the four specific probes for FGF-1 to FGF-4 was performed. The FGF-1 and FGF-2 probes hybridized to the blots (Fig. 2B) but not the FGF-3 or the FGF-4 probes (not shown). One 4-kb FGF-1 and two FGF-2
(2.3 kb and 2.9 kb) transcripts were identified. FGFR-1 mRNA was present before NGF stimulation, and its quantity increased by 2–3-fold during the first 60 min of NGF treatment and subsequently decreased to the initial level. The 2.9-kb FGFR-2 mRNA was present after 10 min of NGF treatment and disappeared after 60 min (Fig. 2B). The 2.3-kb transcript was present in untreated cells, and its amount increased between 30 to 60 min and dramatically decreased between 2 and 6 h. A sudden increase of the 2.9-kb mRNA after 48 h was also observed, and its presence might be required for survival. Reverse transcription-polymerase chain reaction experiments revealed that FGFR-2 exon IIIc was present in PC12 cells before NGF induction, and both IIIb and IIIc exons were present after 72 h of NGF or FGF treatment (data not shown). Activation of FGFRs was assessed in PC12 cells at early times after NGF treatment. Interestingly FGFR-1 as well as FGFR-2 was tyrosine-phosphorylated after 10 min up to 2 h of NGF stimulation (Fig. 2C).

**FGFR Are Involved in NGF-Induced Neuritogenesis—PC12**

**FIG. 1.** FGF-2 binding to NGF-treated PC12 cells. A, 125I-FGF-2 binding to NGF-treated PC12 cells. NGF (30 ng/ml) was added for the indicated time, and binding of 125I-FGF-2 was performed. Open bars (LA) represent “low affinity” binding, and closed bars (HA) represent “high affinity” binding. The values represent the mean ± S.D. from 11 independent experiments. B, cross-linking of 125I-FGF-2 to NGF-treated cells. Cells were treated with NGF as in A and then cross-linked to 125I-FGF-2. 125I-FGF-2 cross-linked material was loaded from samples representing identical cell numbers. The figure shows the autoradiogram (5-day exposure) of 125I-FGF-2-cross-linked material resolved on a 7.5% SDS-PAGE. C, Scatchard analysis results. Increasing amounts of 125I-FGF-2 were bound to NGF-treated or -untreated cells, and binding of 125I-FGF-2 to FGFR receptors was analyzed according to Scatchard (33).

**FIG. 2.** FGFRs expression and activation during NGF-induced neuritogenesis. A, analysis of cross-linked (CL) 125I-FGF-2 from NGF-treated cell lysates immunoprecipitated with anti-FGFR antibodies (αFGFR-1 or αFGFR-2). Autoradiogram (15-day exposure) of 125I-FGF-2 cross-linked to PC12 cells treated by NGF for 0, 6, and 48 h, immunoprecipitated (Ip) with anti-FGFR-1 or anti-FGFR-2 antibodies, and resolved by a 7.5% SDS-PAGE. Five-fold more cells were used (4 × 10⁷ cells) to immunoprecipitate FGFR-2 than to immunoprecipitate FGFR-1. B, FGFRs mRNA expression in NGF-treated cells. The autoradiogram (5-day exposure) of a Northern blot of total RNA extracted at the indicated time and hybridized with FGFR-1 (R1 probe) or FGFR-2 (R2 probe) is shown. The sizes of the detected RNAs are indicated (kb). C, FGFR phosphorylation in NGF-treated cells. Cell lysates were immunoprecipitated (Ip) with anti-FGFR-1 or anti-FGFR-2 antibodies. Immunocomplexes were electrophoresed, immunoblotted (Ib), and revealed with anti-phosphotyrosine antibodies (αPY).
each class of FGFR-DN-expressing cells (DN21, DN24, and DN64) and PCN1 control cells is shown. Experiments described below were done with cells from three clones of each DN receptor expression level (DN21, DN62, and DN63 for high; DN23, DN24, and DN61 for medium; DN22, DN64 and DN65 for low DN expression levels) and two clones harboring the G418-resistance vectors (PCN1 and PCN2). FGFR-1 as well as FGFR-2 was phosphorylated similarly in PC12, and in PCN1 (as shown in Fig. 2C) cells after 10 min of NGF treatment, but in DN21 cells, no phosphorylation was detectable (Fig. 4A). Neurite outgrowth in the presence of NGF for up to 10 days was measured in PC12, PCN1 and 2, DN21, DN24, and DN64 cells. With all of the clones studied, the number of cells with neurites reached a plateau after 96 h (Figs. 3 and 4B). Parental PC12 cells, PCN1, and PCN2 (data not shown) exhibited the same neurite outgrowth pattern. The morphology of PCN1, DN21, DN24, and DN64 was compared (Fig. 3). Neurite outgrowth of DN21 was the most strongly inhibited (70% at 48 h) upon NGF treatment, DN64 was the most weakly inhibited (45%), and DN24 exhibited an intermediary outgrowth pattern (60%); the quantification of this effect was performed by neurite-counting and reported in Fig. 4B. The percentage of cells with neurites did not significantly change over a 10-day period from the percentage estimated at day 4 (data not shown). A total of 11 clones were studied in the neurite outgrowth assay as indicated by Southern blots andautoradiograms of125I-FGF-2-cross-linked (CL) material resolved by 7.5% SDS-PAGE are shown at the right of each figure. Cross-linked DNFGFR-2 is identified by open triangles. The upper band (closed triangles) corresponds to multimeric complexes with possibly endogenous FGF receptors. CL was done with the same number of cells from each clone.

For each experiment, the percentage of cells with neurites at day 4 was calculated and reported in Table I. These data indicate that inhibition of neurite outgrowth was observed in all DNFGFR-2 cell clones and that the degree of inhibition was correlated with the expression of DNFGFR-2. About 76 ± 3.7% of PC12 cells exhibited neurites upon FGF-2 treatment. The neurite outgrowth results were very similar with PCN1 or PCN2 cells. Upon FGF-2 treatment, DN21 and DN24 cells did not differentiate (<1% cells with neurites; not shown), and DN64 exhibited some neurite outgrowth (5–10% of the control; not shown). As other phenotypic differentiation marker, we analyzed peripherin mRNA expression in RNAs isolated from PC12 or DN21 cells after NGF treatment during indicated periods of time (Fig. 4C). PCN1 cells exhibited a 2–3-fold increase in 73 mRNA levels after 12 h (Fig. 4C). The increase in peripherin mRNA level in DN21 cells was moderate and somewhat retarded (24–48 h; Fig. 4C).

FGF Modulates FGF-2 Expression—FGF-2 expression was studied by Western blotting of cell extracts and culture media of cells stimulated by NGF. Practically no FGF-2 was detectable in cell extracts of nonstimulated PC12 or PCN1 control cells. After 5 min of NGF treatment, an 18-kDa FGF-2 isoform was detected in cell extracts (Fig. 5A). FGF-2 was present for up to 96 h with peaks at 1–6 h and at 48–96 h. In DN21 cell extracts, 18-kDa FGF-2 was present in the cell extracts before NGF treatment and disappeared 60 min after (Fig. 5A). In the
TABLE I

Neurite outgrowth assay on different PC12 clones expressing various levels of DN-FGFR2

| DN-FGFR2 expression | 4 days + NGF | 10 days + NGF |
|---------------------|-------------|--------------|
| DN21, DN62, DN63   | 10–15 × basal wt | 29.3 ± 1.86%  |
| DN23, DN24, DN61   | 4–6 × basal wt | 39.7 ± 0.88%  |
| DN22, DN64, DN65   | 1–2 × basal wt | 52.7 ± 1.2%   |
| PCN1, PCN2, PC12wt | NA          | 91 ± 3%       |
| PCN1, PCN2         | NA          | 92 ± 1%       |

Cells were treated with 30 ng/ml NGF for up to 10 days, and the number of cells with neurites was determined after 4 and 10 days. The mean values ± S.E. for these different clones are reported. Expression of the DN-FGFR2 was measured in NGF-induced neuritogenesis was observed in the presence of anti-FGF-2 antibodies, NX-286, or heparin, but NX-213 or scNX-286 did not show any detectable inhibitory effect (data not shown). The mean neurite outgrowth values ± S.E. for different experiments performed three times with cells from different passages after 96 h of NGF stimulation were the following: control, 91.7 ± 1.8%; heparin, 43.3 ± 1.86%; anti-FGF-2 antibodies, 78.3 ± 1.8%; NX-286, 42 ± 0.6%; NX-213, 89 ± 0.6%. These data suggest that autor-crine FGF-2 is implicated in NGF-induced differentiation of PC12 cells.

FIG. 5. FGF-2 expression in NGF-treated PC12 cells. A, determination of the 18-kDa FGF-2 contained in NGF-treated cells. Cell extracts from PCN1 or DN21 DNNGF-F2-expressing cells and conditioned medium from PCN1 cells (PCN-M) were prepared at the indicated time of NGF treatment and immunoblotted as described under “Experimental Procedures.” B, immunoprecipitation of cell surface-associated FGF-2 in NGF-treated cells. Autoradiogram of cell surface-biotinylated anti-FGF-2-immunoprecipitated (Ip) proteins from PCN1 cell extracts without (−) or following (+) 10 or 30 min NGF treatment, resolved by 15% SDS-PAGE.

FIG. 6. Effect of anti-FGF-2 RNA oligonucleotides on NGF-induced neurite outgrowth. PCN1 cells treated with NGF (30 ng/ml (Δ)) were incubated with either 100 μg/ml heparin (○), 200 μg/ml anti-FGF-2 antibodies (△), 100 nm NX-286 FGF-binding-modified RNA oligonucleotide (●), or 100 nm NX-213 vascular endothelial growth factor-specific binding-modified RNA oligonucleotide (□) that were added 10 min before growth factor addition. Squares depict neurite outgrowth assays in which only NGF was added to the medium, as a positive control.
cells were treated or not (NGF (PCN1 and DNFGFR-2 cells. From FGFR in wtPC12 cells, and FGFR-2 (right panel) with anti-FGFR1 (PY) antibodies and chemiluminescence. Arrowheads indicate tyrosine-phosphorylated proteins appearing upon FGF-2 treatment. B, lysates from PCN1 (upper panel) or DN21 (lower panel) cells were incubated with p13-suc-1-agarose, and the proteins were resolved by electrophoresis, immunoblotted, and revealed by anti-Tyr(P) antibodies and chemiluminescence. C, Grb-2/FRS association was assessed in PC12 cells by immunoprecipitation of Grb-2 proteins captured by p13-suc-1-agarose (p13-Ag) beads after release from the Grb-2 immunoprecipitate (upper panel) or immunoblotting with anti-Grb-2 antibodies or anti-phosphotyrosine antibodies (upper panel). D, Shc tyrosine phosphorylation was analyzed by immunoprecipitation of Shc from PCN1 or DN21 cell lysates following or not (U) NGF (N) or FGF-2 (F). Immunocombines were separated by SDS-PAGE, blotted, and revealed by antiphosphotyrosine antibodies (upper panel). The amount of Shc precipitated was assessed by Western blotting with anti-Shc antibodies (lower panel).

over, Shc was tyrosine-phosphorylated in PC12 cells under NGF or FGF-2 treatment, but in PC12DN cells, Shc was found phosphorylated only under NGF stimulation (Fig. 7D, upper panel) with no change in Shc expression (Fig. 7D, lower panel). This result demonstrates that FGFRs are active, whereas endogenous FGFRs are inactivated by the dominant negative FGFR-2.

FGFRs Participate in the Net Sustained ERK Activity Induced by NGF—ERK-1 and ERK-2 activities were determined by phosphorylation of myelin basic protein (MBP). Under NGF stimulation, ERK-1 immunoprecipitates from either PCN1 or DN21 cell extracts presented a similar MBP phosphorylation activity pattern. ERK-1 activity was maximal at 5–10 min and stayed elevated (at about 50% of maximum) for up to 4 h (Fig. 8A). ERK-1 activity in DN21 cell extracts was somewhat higher than in PCN1 cells between 10 and 120 min. ERK-2 activity, however, was 2–3-fold lower in DN21 than in PCN1 cells at 5 min and 7-fold lower 120 min after the beginning of NGF stimulation, and no significant activity was detected after 4 h. ERK-1 and ERK-2 activation were similar in PCN1 cells under NGF or FGF-2 treatment, although the stimulation with the latter was 2-fold lower (Fig. 8A). Furthermore, both ERK-1 and ERK-2 activities were strongly inhibited in DN21 cells following FGF-2 stimulation when compared with control cells (Fig. 8A). Finally, ERK2 activities were similarly low in FGF-2 and NGF-treated DN21 cells. As shown Fig. 8B, the kinetics of MBP phosphorylation by ERK-2 immunoprecipitates as well as ERK-2 phosphorylation were significantly decreased in intensity and length in DN21 cells compared with PCN1, although the amount of protein immunoprecipitated was similar.

**DISCUSSION**

In this study, we show that in PC12 cells, FGFRs, and FGF-2 participate in NGF-induced neuritogenesis. This is based on the following observations. 1) NGF modulates FGFR expression and induces FGFR activation, 2) NGF modulates FGF-2 expression, 3) neurite outgrowth and FGFR activation are reduced in PC12 cells expressing dominant negative FGFR-2, 4) specific anti-FGF-2-modified RNA oligonucleotides inhibit NGF-induced neuritogenesis, and 5) specific FGFR transduction pathways play a crucial role in the maintenance of NGF-induced sustained MAPK activity.

The data presented here revealed the induction of two waves of FGFR expression under NGF treatment. In addition, one FGFR-1 and two FGFR-2 transcripts were identified during NGF stimulation. Numerous FGFR splicing variants have been identified (3, 39, 40). These include variants with either exon IIIb or IIIc usage, two immunoglobulin-like forms, and truncated or soluble FGFRs. Several molecular weight species were present in cross-linking studies, but only a unique complex was detected in either anti-FGFR-1 or anti-FGFR-2 immunoprecipitations. This discrepancy could be explained in part by the...
existence of FGFRs variants truncated at their carboxyl-terminal sequences (41, 42) that are not immunoreactive with the antibodies used. Beside these effects of NGF on FGFR expression, we show here that NGF was able to promote FGFR activation. The functional significance of the FGF receptor activation upon NGF treatment was investigated using the dominant negative FGF receptor approach. Overexpression of dominant negative FGFR-2 reduces the NGF-induced neuritogenesis and reduces the expression of peripherin. Moreover, the key role of SLP/FRS protein in this mechanism was previously reported (24–28, 43). The role of FRS-2 has been clearly demonstrated to participate in the sustained MAPK activity through its interaction with SHP-2 (28). Our data demonstrate also that in PC12DN the MAPK activity is reduced both in intensity and in duration possibly causing the inhibition of differentiation. Because the Shc pathway was affected in DN cells only after FGF-2 treatment, we may conclude that SLP/FRS pathway is probably stimulated only after FGFR activation itself promoted by NGF treatment. The maximal neuritogenesis inhibition we observed was of about 70%, the remaining 30% possibly caused by NGFR activation itself.

The expression of the FGF-2 was stimulated by NGF in PC12 cells and found both in cell extracts and on the cell surface. We demonstrate here that newly produced FGF-2 mainly stays on the cell surface to act on its receptors. The feeble inhibitory effect of FGF-2-neutralizing antibodies has been already noted in previous studies (47) possibly because of their size which does not allow access to membrane- or extracellular matrix-bound FGF-2. However, when using anti-FGF-2 RNA oligonucleotides that specifically neutralize extracellular FGF-2 (31), neuronal differentiation induced by NGF was inhibited at about 50%. This implies the existence of an NGF-driven FGF-2 autocrine loop. The activation of the FGFRs by endogenous FGF-2 is not incompatible with the participation of other FGFR ligands in neuritogenesis. For example, FGF-1 is expressed during differentiation of PC12 cells and possibly participates in FGFR-1 activation (48) or the adhesion molecule L1 signals through FGFRs and induces neuronal differentiation of neu-
rons in culture (49). FGF-2 knock-out mice show some abnormalities in the cytoarchitecture of the neocortex and significant reduction in neuronal density in most layers of the motor cortex (50). Moreover, neuronal defects are present in the hippocampal commissure, and neuronal deficiencies are observed in the cervical spinal cord. Furthermore, FGF-2 knock-out mice present an impaired neural regulation of blood pressure by the baroreceptor reflex, suggesting a role for the sympathetic system (51). Therefore, the interpretation of our results in that context suggests that FGF-2 may be important for neuronal differentiation especially in the case of sympathetic neurones, modeled by PC12 cells.

The results described in the present work indicate that FGFRs are involved in neuronal differentiation of PC12 cells induced by NGF and that MAPK-sustained activation is dependent on functional FGFR system. Furthermore, autocrine FGF-2 participates in NGF-mediated FGFR activation. It is possible that the mechanism of NGF action described herein is specific for a population of sympathetic neurones and regulate their function. But also, this mechanism could have a general significance and operate in different neuronal cell populations, thus placing FGFRs in or alongside the NGF signal transduction pathway.

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