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NGF Induces Transient But Not Sustained Activation of ERK in PC12 Mutant Cells Incapable of Differentiating

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Abstract Activation of receptor tyrosine kinases stimulates a diverse array of cellular responses such as proliferation and differentiation. The first events in the signal transduction pathways mediated by different receptor tyrosine kinases are similar and include activation of the mitogen-activated protein kinase (MAPK) pathway and the induction of immediate early genes. The precise signaling pathways leading to each of the cellular responses mediated by receptor tyrosine kinases are still unknown, although it has been proposed that sustained activation of the MAPK pathway by receptor tyrosine kinases such as the nerve growth factor (NGF) receptor TrkA is sufficient to induce differentiation in PC12 cells. In the present study we examined the effect of NGF on mutant PC12 cells that were derived spontaneously in our cultures. NGF induced normal activation of immediate early genes in these cells, whereas the activation of some delayed response genes, as well as neurite outgrowth, was impaired. Furthermore, activation of the NGF-induced extracellular signal-regulated kinase (ERK) in these cells was transient, not sustained. These results support the hypothesis that sustained activation of ERK plays an important role in activating the induction of delayed response genes. However, sustained ERK activation is not a mandatory condition for the promotion of all the features of differentiated PC12 cells, as NGF could induce transcription of the delayed response gene, transin, in PC12 mutant cells. Taken together, our results suggest that NGF induces differentiation of PC12 cells via several signaling pathways, an important one of which is the MAPK pathway. J. Cell. Biochem. 70:425–432, 1998.

Key words: nerve growth factor; tyrosine kinase receptors; differentiation; PC12 cells; mitogen-activated protein kinase

Peptide growth factors play a critical role in the development and maintenance of the nervous system. Binding of various growth factors to their receptor tyrosine kinases (RTKs) mediates a diverse array of cellular responses; some of which lead to differentiation and others to proliferation. The response of the rat pheochromocytoma cell line PC12 [Greene and Tischler, 1976] to RTK activation has been extensively used as an experimental system to study the mechanisms of these diverse responses. Treatment of PC12 cells with nerve growth factor (NGF) or fibroblast growth factor (FGF) leads to their differentiation into postmitotic cells resembling sympathetic neurons [for review see Fujita et al., 1989; Teng and Greene, 1994]. The effect of NGF on PC12 cell differentiation is mediated via the NGF-receptor tyrosine kinase TrkA, and requires the activation of specific programs of immediate early gene (IEG) and delayed response gene (DRG) expression. Some of the IEGs encode transcription factors that probably control the expression of NGF-specific DRGs, which in turn encode primary structural proteins and enzymes that contribute to the neuronal phenotype of NGF-treated PC12 cells [for review see Chao, 1992; Greene and Kaplan, 1995; Segal and Greenberg, 1996; Kaplan and Miller, 1997]. Treatment of PC12 cells with epidermal growth factor (EGF) activates the EGF-receptor tyrosine kinase, which in contrast to TrkA activation leads to a proliferative signal [Huff et al., 1981]. Detailed analysis and comparisons of the signaling pathways induced by NGF and EGF reveal that both factors ini-

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tially induce very similar signal transduction pathways [Chao, 1992] and programs of IEG transcription [Sheng and Greenberg, 1990]. However, they induce different DRG programs. The mechanism whereby RTKs transduce a signal from the plasma membrane to the nucleus to activate programs of IEG transcription involves the activation of the Ras mitogen-activated protein kinase (MAPK) pathway, which includes activation of Ras and sequential phosphorylation of Raf, MEK (MAPK or extracellular signal-regulated kinase [ERK] kinase), MAPK, or ERK, and ribosomal protein S6 kinase [for review see Seger and Krebs, 1995; Kaplan and Miller, 1997; Robinson and Cobb, 1997]. IEG transcription is induced, at least in part, by translocation of the activated ERK into the nucleus, where it phosphorylates and activates nuclear transcription factors such as Elk-1. Activated Elk-1, together with other transcription factors, binds to the promoters of IEG and induces their transcription [for review see Davis, 1995; Segal and Greenberg, 1996; Karin et al., 1997]. As both NGF and EGF initially induce similar signal transduction events, but stimulate different cellular responses (e.g., different DRG programs), a key question concerns the point of bifurcation of the routes for NGF and EGF signaling. Experiments in PC12 cells have shown that NGF and EGF differ with respect to the duration of the ERK activation that they induce. NGF induces sustained activation, whereas EGF induces transient activation of ERK [for review see Marshall, 1995; Greene and Kaplan, 1995]. Their experiments led Marshall [1995] to propose the model which suggests that the duration of ERK activation is critical for determining whether RTK activation will lead to differentiation or proliferation.

In the present study we describe PC12 mutant cells in which NGF induces ERK activation that is transient, not sustained. Furthermore, it appears that the induction of IEG by NGF is normal in these cells, whereas the induction of DRG is impaired. These results imply that the NGF-dependent induction of some DRGs requires sustained ERK activation, and support the hypothesis that sustained ERK activation plays an important role in mediating the differentiation of PC12 cells. In addition, although the induction of some DRGs by NGF is impaired, the induction of transin seems normal. Our results therefore suggest that there are multiple signaling pathways in the NGF-mediated induction of DRGs.

MATERIALS AND METHODS

Materials

NGF (2.5S) was purchased from Chemon International (Harrow, UK). [^{125}I] 2.5S NGF (1,500 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). EGF was purchased from Sigma (St. Louis, MO).

Cell Cultures

PC12 and PC12m cells were grown in RPMI 1640 supplemented with heat-inactivated 10% horse serum and 5% fetal calf serum on rat tail collagen-coated plates (Biological Industries, Beit Ha'emek, Israel).

Isolation of PC12m Cells

PC12m cells, which have a distinct flattened morphology, adhere very strongly to the substrate and proliferate much faster than the parental PC12 cells. These features were used to separatethe PC12m cells from the parental PC12 cells. First, a cell population enriched in PC12m cells was obtained by treatment of cultures with EGTA (0.5 mM); this procedure was sufficient to detach parental but not PC12m cells from the substrate. Purified PC12m cells were subsequently obtained by two sequential subcloning procedures.

[^{125}I] NGF Binding

Binding of [^{125}I]-NGF was carried out in intact 3- to 4-day-old cultures of PC12 or PC12m cells growing on 24-well plates. (Cultures were nonconfluent at this stage.) Prior to each experiment, the serum-containing growth medium was gently replaced with serum-free medium supplemented with 0.1 mg/ml bovine serum albumin (BSA). Binding experiments were carried out in 0.2 ml RPMI medium containing 0.1 mg/ml BSA and 30 pM [^{125}I]-NGF in the presence or absence of the indicated concentration of unlabeled NGF for 18 h at 4°C. Binding was terminated by placing the plates on ice and gently rinsing three times with RPMI medium containing 0.1 mg/ml BSA. Cells were subsequently lysed with 1% SDS and counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 5 nM unlabeled NGF. All measurements were carried out in quadruplicate. The apparent inhibition con-

stant of NGF for the high-affinity binding sites was extracted from the inhibition curve.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from the cells by the lithium chloride precipitation method of Cathala et al. [1983]. For Northern blot analysis, 25-µg aliquots of total RNA were resolved by electrophoresis through a 6.5% formaldehyde/1% agarose gel, and capillary-transferred with 10µ saline-sodium citrate (SSC) to a Gene Screen membrane (New England Nuclear, Boston, MA). Probes were ^{32}P-labeled by nick translation. Blots were hybridized in 50% formamide, 6µ SSC, 1µ Denhardt's solution, 50 mM Tris-HCl (pH 7.5), 1% SDS, 10% dextran sulfate, and 200 µg/ml of salmon sperm DNA at 42°C overnight. Blots were washed to a final stringency of 68°C in 0.2µ SSC, 0.2% SDS for 1 h. Washed membranes were exposed for 48 h at -70°C to Agfa Curix RP2 X-ray films with intensifying screens. After every hybridization, each blot was rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to verify the equality in RNA loading.

DNA Probes

SCG10 is an 800-bp *Pvu*II-*Eco*RI fragment from clone SCG10-6 [Stein et al., 1988]; neurofilament (NF)-L is a 300-bp *Eco*RI fragment from pNF-L; NF-M is a 600-bp *Eco*RI fragment from pNF-M [Lindenbaum et al., 1988]; transin is a 1600-bp *Eco*RI fragment of pTR1 [Machida et al., 1989]; c-fos is a 1000-bp PstI fragment from p-fos 1 [Sassone-Corsi et al., 1988]; c-myc is a 900-bp PstI fragment from pCmyc54 [Gasin et al., 1984]; junB is a 1700-bp *Eco*RI fragment from pjunB [Wu et al., 1989]; TIS-8 is a 900-bp *Eco*RI fragment from pTIS8 [Altin et al., 1991]; GAPDH is a 1100-bp Pst-1 fragment from pRGAPDH-1 [Piechaczyk et al., 1984]; SCG4 is a 2,000-bp *Eco*R1 fragment from pSP6SCG4 [Anderson and Axel, 1985].

Determination of ERK Activity

ERK2 activity was determined as previously described [James et al., 1994]. Briefly, PC12 or PC12m cells were plated at a density of 1×10^6 cells/30-mm dish in serum-free medium for 24 h (these particular PC12 subclones are relatively resistant to serum deprivation). Cells were stimulated for 5, 10, 15, 30, 60 or 120 min by the addition of EGF (10 ng/ml) or NGF (50 ng/ml). ERK activity was then assayed in the cell lysates. The medium from each dish was replaced with 1 ml of ERK lysis buffer (50 mM -glycerophosphate, 1.5 mM EGTA, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM benzamidine, and 1% NP-40), and the dishes were kept on ice for 10 min. The cell lysates were then collected into eppendorf tubes, vortexed, and centrifuged at 10,000*g* for 10 min at 4°C, and the supernatants were collected. Samples containing 1 mg protein were then incubated for 2 h at 4°C with 5 µl of anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and 30 µl of protein A-sepharose beads (Sigma) diluted 1:1 in water. The supernatant was discarded and the beads were washed twice with ERK lysis buffer, followed by a wash with kinase buffer (30 mM Tris-HCl pH 8.0, 20 mM $MnCl_2$, 2 mM $MgCl_2$). The beads were incubated for 30 min at 37C in 30 µl of complete kinase buffer (containing the above kinase buffer and 15 µg myelin basic protein (MBP) 10 M ATP and 5µ Ci γ^{32}P-ATP, 3,000 Ci/mmole). The reaction was stopped by the addition of 6 µl of six Laemli sample buffer and heating for 5 min at 95°C. Following a short spin, the supernatants were collected and the proteins separated by SDS-polyacrylamide gel electrophoresis 15% acrylamide on mini-gels. Phosphorylated MBP was visualized by autoradiography of the dried gels on X-ray films and quantified by densitometric analysis. The relative ERK2 activity was normalized to the levels of MBP (measured following protein staining of the gel).

RESULTS

Isolation and Morphological Characterization of PC12m Clones

During continuous culturing, PC12 cells, like many other cell lines, are subject to spontaneous mutations that lead to the generation of PC12 cell variants [Teng and Greene, 1994]. A variant clone was isolated from continuously growing PC12 cultures and was termed PC12m. As shown in Figure 1, PC12m cells are morphologically distinct from their parental PC12 cells. The parental cells are small round cells, whereas the variant cells are large and flattened, and extend small cytoplasmic extensions. In addition, the adhesiveness of PC12m

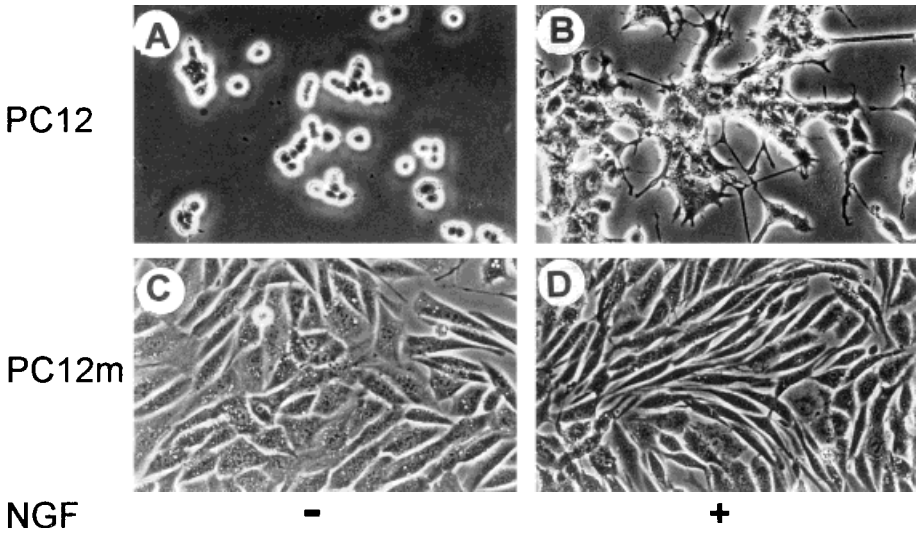

Fig. 1. NGF does not induce neurite outgrowth in PC12m cells. 1 x 10^4 cells/well of PC12 (**A,B**) or PC12m (**C,D**) cells were grown on collagen-coated six-well plates for 3 days in the presence (B,D) or absence (A,C) of 50 ng/ml NGF. The photographs were taken with a phase-contrast microscope, magnification ×200.

cells to the substrate is higher than that of the parental cells.

To further characterize the morphological features of PC12m cells, we examined their morphological responses to NGF, which induces neurite outgrowth in parental PC12 cells. As shown in Figure 1, addition of NGF to PC12m cells for 3 days (Fig. 1D) or up to 7 days (not shown) did not induce either neurite outgrowth or morphological changes.

Expression of NGF Receptors

To determine whether high-affinity NGF receptors are present in PC12m cells, we measured the binding of [^{125}I]-labeled NGF to intact cells. As shown in Table 1, PC12m cells contain high-affinity NGF-binding sites and their levels, although lower (by about 43%), are comparable to those of parental PC12 cells.

A competition experiment employing 30 pM [^{125}I]-NGF and various concentrations (0.03 to 1 nM) of unlabeled NGF revealed similar inhibition constants of NGF in PC12m and parental PC12 cells (0.36 and 0.26 nM, respectively).

Abbreviations: BSA, bovine serum albumin; CREB, cAMP regulatory element-binding protein; DRG, delayed response gene; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IEG, immediate early gene; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NF, neurofilament; NGF, nerve growth factor; RTK, receptor tyrosine kinase; SSC, saline-sodium citrate.

TABLE I. Measurement of [^{125}I]-NGF Binding to PC12 Cells[a]

Cells	Number of high-affinity NGF-binding sites (fmol/mg protein)
Parental PC12	1.13 ± 0.11
PC12m	0.65 ± 0.04

[a]Specific binding of [^{125}I]-NGF (30 pM) to intact PC12 cells was carried out at 4°C for 18 h. Under these conditions [^{125}I]-NGF detects only high-affinity binding sites [Buxser et al., 1990], and only surface-bound [^{125}I]-NGF is measured and internalization does not occur [Bernd and Greene, 1984]. Data are means ± SD of quadruplicate determinations from a representative experiments (one of four independent experiments with similar results).

Taken together, these results suggest that the levels and properties of the high-affinity NGF receptors in PC12m cells are comparable to those in parental PC12 cells.

NGF Induces IEG Transcription in PC12m Cells

Previous studies have shown that NGF induces rapid and transient expression of IEGs such as c-fos, c-jun, jun-B, TIS8/NGF1A and c-myc in PC12 cells [for review see Sheng and Greenberg, 1990]. Maximal induction is reached within 30 to 60 min, except in the case of c-myc, which reaches maximal induction 4 h following stimulation. Accordingly, in an attempt to further determine the responsiveness of PC12m to NGF we examined the effect of NGF on IEG transcription. As shown in Figure 2, NGF in-

duced rapid and transient transcription of these genes (as represented by c-jun), like that induced in parental PC12 cells [Greenberg et al., 1986; Wu et al., 1989; Altin et al., 1991] (Fig. 2C, as shown for c-fos).

Induction of DRGs

Since NGF signaling induces IEG transcription in PC12m cells, we next examined whether this signaling can be propagated further, leading to induction of DRG transcription. The genes examined were SCG10, transin, NF-L and NF-M, as previous studies have shown that these genes are induced in parental PC12 cells following treatment for 24 h with NGF [Stein et al., 1988; Machida et al., 1989; Lindenbaum et al., 1988]. As shown in Figure 3B, treatment of PC12m cells with NGF for 24 h resulted in an increase in the levels of transin mRNA, but not of SCG10, NF-L, or NF-M mRNAs. In parental PC12 cells, however, such treatment resulted in an increase in the levels of NF-L, NF-M, and SCG10 mRNAs (Fig. 3A).

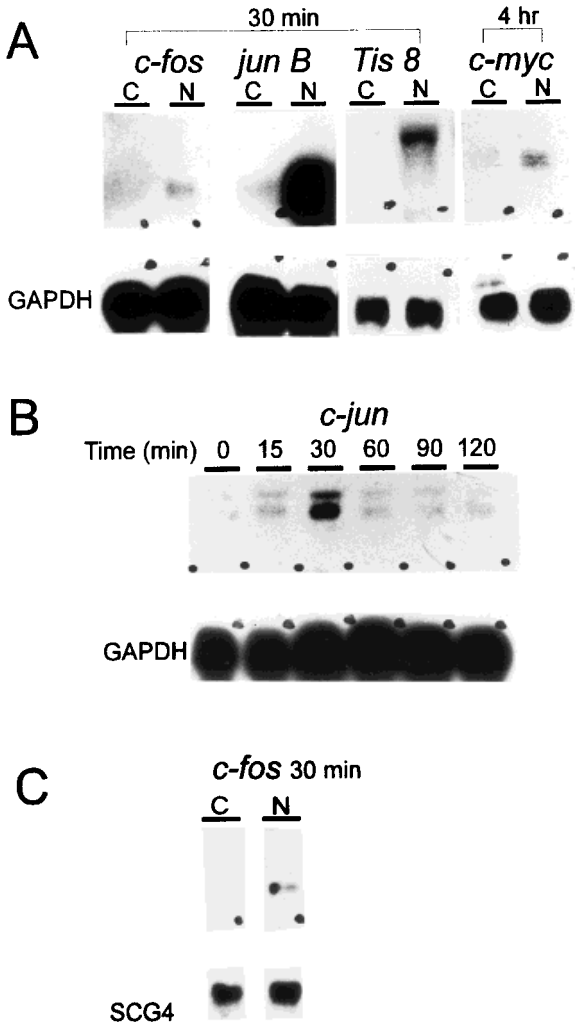

Fig. 2. NGF induces IEG transcription. PC12m (**A,B**) and PC12 (**C**) cells were treated with (N) or without (C) 50 ng/ml NGF for the indicated times. Total RNA (25 μg) extracted from each treatment was subjected to Northern blot analysis. Blots were probed with ^{32}P-labeled probes for the indicated genes. These blots were then dehybridized and reprobed with GAPDH or SCG4 to reveal variations in sample loading. A time course of c-jun induction is shown in B. Dots indicate the position of the 18S r-RNA.

NGF Induces Transient Activation of ERK

Differentiation of PC12 cells is accompanied by sustained ERK activation, suggesting that prolonged activation of the MAPK pathway determines the response of the cells with respect to differentiation. In view of our finding that in PC12m cells NGF signaling stimulates a normal pattern of IEG expression but impaired expression of DRGs, we were interested in finding out whether the inability of NGF to induce complete differentiation of PC12m cells could be a reflection of its effect on the kinetics of ERK activation. We therefore examined the kinetics of NGF-induced ERK activation in PC12m cells. ERK2 was immunoprecipitated from solubilized PC12m cells stimulated with either NGF or EGF for up to 120 min. The protein kinase activity of the immunoprecipitated ERK2 was assessed using MBP as substrate. Figure 4 shows that in parental PC12 cells NGF induced activation of ERK2 enzymatic activity which was sustained for at least 120 min, whereas the ERK2 activation induced by EGF was transient. In contrast, the ERK2 enzymatic activity induced by NGF in PC12m cells was transient, with similar kinetics to those observed for EGF.

These results therefore demonstrate that PC12m cells, like the parental PC12 cells, can respond to NGF signaling by activating the MAPK pathway. However, PC12m cells exhibit impairment of the kinetics of ERK activation, in that there is a change from prolonged to transient activation.

DISCUSSION

A key issue in studying signaling pathways activated by neurotrophic factors is to understand the mechanism underlying neuronal differentiation. Previous studies have increased our understanding of the intracellular signaling pathways induced by RTK activation. In

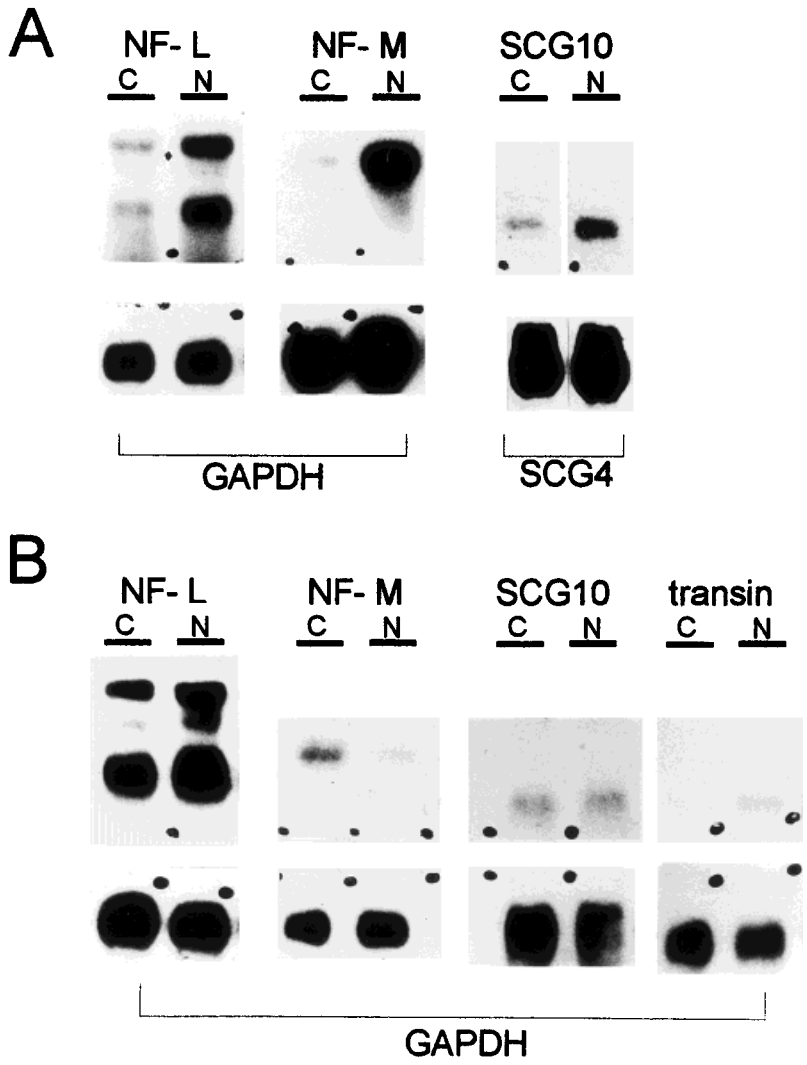

Fig. 3. NGF induces transcription of transin but not of NF-L, NF-M, or SCG10. PC12 (**A**) and PC12m(**B**)cells were treated with (N) or without (C) NGF for 24 h. The levels of mRNA expression of the indicated genes were analyzed by Northern blots as described for Figure 2. The dots indicate the position of the 18S r-RNA.

many of these studies, neurite outgrowth was used as an indicator for neuronal differentiation. However, as previously discussed [Greene and Kaplan, 1995], neuritogenesis is only one manifestation of neuronal differentiation. Moreover, neurite outgrowth may not be an adequate indicator of differentiation: in at least some cases in which discrete signaling mechanisms were proclaimed to result in neuritogenesis, i.e., differentiation, these responses turned out to be merely an extension of short cytoplasmic process and not the stable and extensive networks generated in response to neurotrophins [Greene and Kaplan, 1995].

Accordingly, in the present study we examined the effects of NGF on neurite outgrowth as well as on the induction of IEGs and DRGs, and correlated these effects with the kinetics of NGF-induced ERK2 activation in PC12m cells. We show that the induction of IEGs (e.g., c-jun, c-fos, jun B, Tis 8, and c-myc) by NGF appeared normal in PC12m cells, whereas the induction of DRGs (e.g., SCG10, NF-M, and NF-L) and of neurite outgrowth were impaired. Furthermore, the NGF-induced ERK activation was transient rather than sustained, i.e., the activity reached a peak after 10 min and then decreased rapidly. The activity did not decline to the basal level, however, and some residual activity was still observed after 120 min. Although we cannot exclude the possibility that this residual activity might still be sufficient to

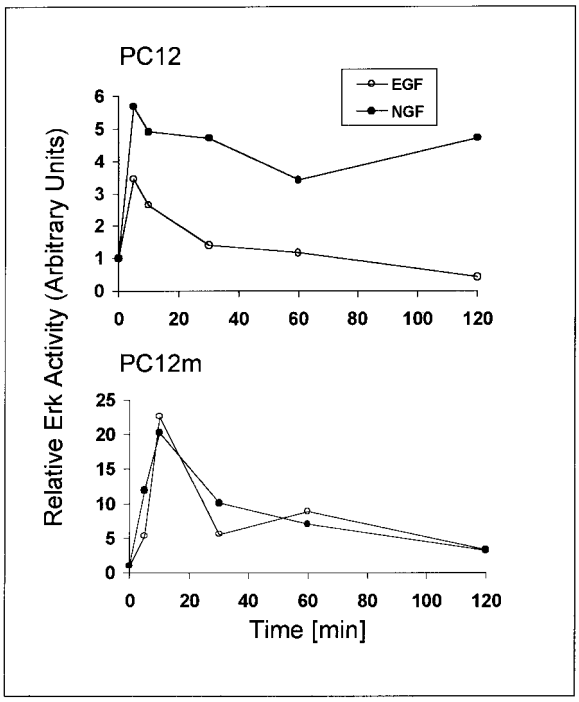

Fig. 4. Activation of ERK2 in PC12 and PC12m cells following stimulation by NGF and EGF. PC12 or PC12m cells were stimulated with either NGF (●) or EGF (○) for the indicated times and the specific activity of ERK2 was measured as described in Materials and Methods. The data presented are from a representative experiment (one of three independent experiments with similar results).

mediate NGF-induced differentiation, we think this unlikely, as EGF-induced ERK activity in PC12m cells exhibited similarly transient kinetics. Moreover, this activity also did not decline to the basal level, and its level after 120 min was almost the same as that of NGF-induced activity.

These results may thus support the hypothesis that the sustained activity of MAPK pathways plays an important role in determining whether differentiation will occur, and suggest that PC12m cells are defective in the mechanism that leads to NGF-induced sustained ERK activation. The mechanism whereby sustained activation of the MAPK pathway leads to induction of DRGs is not known. It was proposed [Segal and Greenberg, 1996] that the cAMP regulatory element-binding protein (CREB) as well as IEGs may regulate the transcription of at least some DRGs that contain CREB-binding sites within their promoters. NGF and other growth factors stimulate phosphorylation of CREB at Ser133 by the ribosomal protein S6 kinase via the MAPK pathway [Xing et al., 1996]. NGF induces a sustained CREB Ser133 phosphorylation, whereas the phosphorylation stimulated by EGF is only transient [Ginty et al., 1994; Bonni et al., 1995]. It was therefore suggested that CREB and IEGs are required for the transcription of DRGs and that by the time the IEG protein products are formed, CREB is still activated in NGF-treated cells but not in EGF-treated cells [Segal and Greenberg, 1996].

Although several observations support the hypothesis that the prolonged activation of the MAPK pathway determines whether or not differentiation will occur, it seems that this pathway by itself is not mandatory to evoke all the differentiation responses. For example, persistent activation of the MAPK pathway alone was insufficient to induce differentiation in PC12 cells expressing mutant platelet-derived growth factor receptors [Vaillancourt et al., 1995], and expression of mutant activated Ras in PC12 cells was not sufficient (although necessary) for induction of calcium channel [Pollock and Rane, 1996]. These reports as well as others [Kaplan and Miller, 1997] suggest that other signaling pathways beside the sustained MAPK pathway act to induce the multiplicity of neuronal differentiation responses. This notion is also supported by our results, as although NGF induced only transient ERK2 activation it could induce transin mRNA transcription.

The precise nature of the defect in PC12m cells that impairs the NGF-dependent induction of prolonged ERK activation and the transcription of SCG10, NF-L and NF-M genes is not known. One possible explanation is that the cells have an impaired endocytosis mechanism, resulting in a 43% reduction in the number of high-affinity NGF-binding sites. We have observed, however, that FGF (which is also a differentiation factor for PC12 cells) exerts exactly the same effects as NGF on PC12m cells, i.e., FGF induces normal IEG expression but impaired DRG expression as well as transient ERK2 activation (data not shown), despite the fact that the numbers of FGF binding sites are similar in parental and PC12m cells [Stein and Neufeld, unpublished results]. It thus appears that the defect which impairs NGF and FGF responses in PC12m cells does not reside in the small reduction in high-affinity NGF receptor levels, but downstream from the RTK and their immediate substrates. Such sites could be phosphatases that dephosphorylate the different ki-

nases in the MAPK pathway. Whether defects in these phosphatases or inother molecules contribute to the impaired responsiveness of PC12m cells to NGF remains to be determined.

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