Schwannoma-derived Growth Factor Promotes the Neuronal Differentiation and Survival of PC12 Cells

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Abstract. Schwannoma-derived growth factor (SDGF) was initially isolated from schwannoma cells as a mitogen for glial cells and fibroblasts. The present data show that SDGF causes the morphological and molecular differentiation of rat PC12 cells in a manner similar to, but distinguishable from nerve growth factor (NGF). It also promotes PC12 survival in serum-free conditions. SDGF induced changes include neurite outgrowth and the induction of the mRNAs for GAP-43 and transin, proteins which are highly expressed in axons. In addition, both SDGF and NGF induce the transcription factor, NGFI-A. The time course of the response to SDGF is similar to that for NGF. Gap-43 mRNA induction by both SDGF and NGF is inhibited by dexamethasone, but dexamethasone has no effect on NGFI-A mRNA synthesis. These observations show that SDGF has a differentiation and survival promoting effect on PC12 cells in addition to its mitogenic activity on glial cells and fibroblasts.

SCHWANNOMA-derived growth factor (SDGF)[1] was originally isolated from the growth-conditioned medium of a schwannoma cell line based on its growth-potentiating activity for astrocytes and fibroblasts (Kimura et al., 1990). In addition, SDGF weakly potentiates the proliferation of Schwann cells. It belongs to the EGF family of growth factors and is expressed in several tissues including embryonic brain and sciatic nerve (Kimura et al., 1990), suggesting an involvement in the development of both central and peripheral nervous systems.

Several peptide growth factors which were originally identified as mitogens have recently been shown to function also as survival and/or differentiation factors. EGF, a potent mitogen for cells of ectodermal and mesodermal origin, alters the phenotypic characteristics of cultured rat pituitary cells (Johnson et al., 1980). Fibroblast growth factor (FGF), first identified as a mitogen for fibroblasts, is also a differentiation factor for a variety of neuroectoderm derived cells (see review Baird and Bohlen, 1990). Conversely, nerve growth factor (NGF), initially characterized as a differentiation and survival factor, is mitogenic for certain cells (Lillien and Claude, 1985).

Until recently the study of the differentiation promoting effects of growth factors has been hampered by the difficulty in quantitating the morphological and molecular changes. However, several neuron-specific proteins whose expression is coincident with differentiation have now been purified and cloned. Two such differentiation markers as well as the early response gene were used in the following experiments: GAP-43, transin, and NGFI-A. The growth-associated protein, GAP-43 (also called B50), was first identified as a synaptosomal plasma membrane protein and was later found in growing axons (Skene and Willard, 1981*a*,*b*; Zwiers et al., 1978, 1982, 1985). This protein is axonally transported and is abundant in growth cones (Meiri et al., 1986; Skene et al., 1986). GAP-43 protein and mRNA levels increase rapidly just before axon extension in cultures of sympathetic ganglia (Meiri et al., 1988), primary hippocampus (Goslin et al., 1988, 1990), dorsal root ganglion (Woolf et al., 1990), and PC12 cells treated with NGF (Van Hooff et al., 1986; Basi et al., 1987; Federoff et al., 1988; Costello et al., 1990). NIH 3T3 and COS cells transfected with GAP43 elaborate a variety of filopodial extensions reminiscent of neurites (Zuber et al., 1989). Pheochromocytoma (PC12) cells stably transfected with GAP-43 extend neurites in a more rapid fashion in response to a 10-fold lower concentration of NGF than the wild type PC12 cells (Yankner et al., 1990). These observations indicate that GAP-43 plays an important role in neurite extension and provides a molecular marker for this process.

Proteases are released at the growth cones of developing neurons in culture (Krystosek and Seeds, 1981*a*,*b*; Pittman, 1985), suggesting their involvement in axon elongation (see reviews Patterson, 1985; Monard, 1988). Transin is a secreted metalloprotease of 53,000 daltons. It is induced in PC12 cells by NGF or FGF, and the time course of transin induction is coincident with the neuronal differentiation of these cells (Machida et al., 1989).

NGFI-A is a gene originally discovered in PC12 cells based on its induction by NGF (Milbrandt, 1987). This gene product belongs to a family of zinc finger proteins whose

1. *Abbreviations used in this paper*: FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; MTA, 5'-S-methyl adenosine; NGF, nerve growth factor; PC, pheochromocytoma cell; TGF, transforming growth factor.

archetype is the Xenopus transcription factor TFIIIA (Ginsberg et al., 1984). NGFI-A is expressed in many tissues including the rat nervous system during embryonic and postnatal development (Watson and Milbrandt, 1990).

The following paragraphs show that SDGF potentiates the survival and induces neurite outgrowth of PC12 cells as well as the mRNAs for neurite marker proteins GAP-43 and transin in a dose-dependent manner. mRNA for NGFI-A is also induced by SDGF. These gene products were all induced by SDGF with a time course similar to NGF, suggesting that SDGF causes the differentiation of PC12 cells.

Materials and Methods

Cell Culture

PC12 cells were grown in DME supplemented with 10% FCS and 5% horse serum on 10-cm tissue culture dishes which were precoated with 50 ng/ml of poly-L-lysine. After reaching ~30% confluence, cells were washed and cultured in N_2 serum-free defined medium (Bottenstein and Sato, 1979) and then NGF (10 ng or 4 ng/ml), or SDGF (10 ng/ml) were added to the medium. Cells were harvested 1, 2, 4, 8, 12, 24, 48, 72, and 96 h after stimulation for RNA blot analysis.

RNA Blot Analysis

Total cellular RNA was isolated by following the procedure of Chomczynski and Sacchi (1987). Total RNAs (10 μg/lane) were electrophoresed in a 0.66 M formaldehyde denaturing gel and blotted on Hybond-N nylon. Hybridization was performed in a solution of 50% formaldehyde, 50 mM sodium phosphate buffer (pH 6.5), 1 × Denhardt's solution, 5 × SSC, 100 μg/ml salmon sperm DNA at 42°C for 16 h with probes labeled with ^{32}P by multiprimer labeling kit (Amersham Corp., Arlington). After hybridization, the membranes were washed briefly in 0.1 × SSC, 0.2% SDS at room temperture, followed by 30 min washes twice in 0.1 × SSC, 0.2% SDS at 65°C. The hybridization membranes were exposed with intensifying screens for 1–2 d at −70°C. Blots were scanned on an Ultroscan (LKB Instruments Inc., Bromma, Sweden). All northern blot experiments were done at least twice with similar results.

Chemicals and Reagents

GAP43 cDNA was obtained from Dr. Pate Skene (Stanford University, Stanford, CA) transin cDNA from Dr. Lynn Matrisian (Vanderbilt University, Nashville, TN), and NGFI-A cDNA from Dr. Jeffery Milbrandt (Washington University, St. Louis, MO). EGF and NGF were purchased from Upstate Biologicals and dissolved in DME. Dexamethasone, cycloheximide, and 5'-S-methyl adenosine were purchased from Sigma Chemical Co. (St. Louis, MO). SDGF was prepared from the growth condition medium of the JS1 schwannoma cell line as previously described by Kimura et al. (1990) and its concentration determined by amino acid analysis.

Results

Morphology and Neurite Outgrowth

The morphological responses of PC12 cells to SDGF are very similar to those to NGF. To eliminate the synergetic effects of serum, we used the chemically defined N_2 medium (Bottenstein and Sato, 1979) and polylysine-coated dishes (Schubert et al., 1987). In this medium the onset of neurite extension caused by NGF and SDGF is faster than that in serum-supplemented medium. Both SDGF and NGF induce the production of short spikes within 3 h following their addition to cultures. Neurites, which are distinguishable from short spikes in that they possess flattened growth cones, are detectable after 8 h and are present for up to 5 d in both SDGF and NGF (Figs. 1 and 2). The rate of neurite extension is the same for NGF and SDGF for the first 24 h, but after this time neurite length in the presence of SDGF remains relatively constant while that caused by NGF increases (Fig. 2). Daily addition of SDGF to the cultures did not induce additional growth. These data indicate that SDGF induces the initial stages of neurite outgrowth in sympathetic-like nerve cells.

Survival in Serum-free Medium

PC12 cells are not viable in medium lacking serum. However, supplementation of serum-free medium with NGF promotes the survival of PC12 cells. To compare the survival activity of SDGF with that of NGF, cells were plated in serum-free medium in the presence of increasing amounts of these growth factors. After 24 h the optimal survival was observed at 10 ng/ml of SDGF, with half-maximal effects at 5 ng/ml (Fig. 3). The half maximal survival activity of NGF was at an approximately 10-fold lower protein concentration. After 48 h a greater fraction of the input cells died in the presence of SDGF than with NGF. These data show that SDGF has a dose-dependent survival activity on PC12 cells.

Induction of GAP-43, Transin, and NGFI-A mRNAs by SDGF

Since the initial morphological changes in PC12 cells caused by SDGF and NGF are similar, the effects of both growth factors were evaluated on the induction of the marker genes GAP-43, transin and NGFI-A. GAP-43 cDNA was cloned from neonatal rat brain (Basi et al., 1987) and was used for Northern blot analysis. GAP-43 mRNA was expressed at a low level even in the absence of SDGF or NGF (Fig. 4, *c*). The mRNA started to increase by 4 h following SDGF and NGF treatment. In the presence of SDGF, GAP-43 mRNA reached its maximal level at 8 h and maintained this high level of expression for 24 h before falling. GAP-43 mRNA in cells treated with NGF continued to increase for 2 d, then decreased to the control level (Fig. 4, *c* and *g*).

To date, NGF and FGF are the only growth factors capable of inducing transin mRNA in PC12 cells (Machida et al., 1989). Northern blot analysis showed that SDGF also induced the 1.9-kb transin mRNA (Fig. 4, *b*). Transin mRNA started to be expressed at 4 h after the treatment with SDGF or NGF. With SDGF, it increased and reached a peak at 12 h and then abruptly declined. After NGF treatment, transin mRNA was also maximally expressed between 4 and 12 h after the application of NGF (Fig. 4, *b* and *f*). The kinetics of transin and GAP-43 presented here are somewhat different than those presented in the original reports (Machida et al., 1989; Costello et al., 1990), but the original studies were done in different culture conditions from ours. The above experiments were done in N_2 medium with poly-L-lysine–coated dishes.

NGFI-A was cloned as an immediate early response gene from PC12 cells treated with NGF by differential hybridization (Milbrandt, 1987). Fig. 4 *a* shows NGFI-A mRNA was expressed at a maximal level at 1 h after treatment with SDGF, then decreased gradually, and was barely detected at 12 h after the application of SDGF. In contrast, NGFI-A was detectable up to 2 d later in cells treated with NGF. The maximum induction by NGF was threefold higher than that by SDGF (Fig. 4, *a* and *e*).

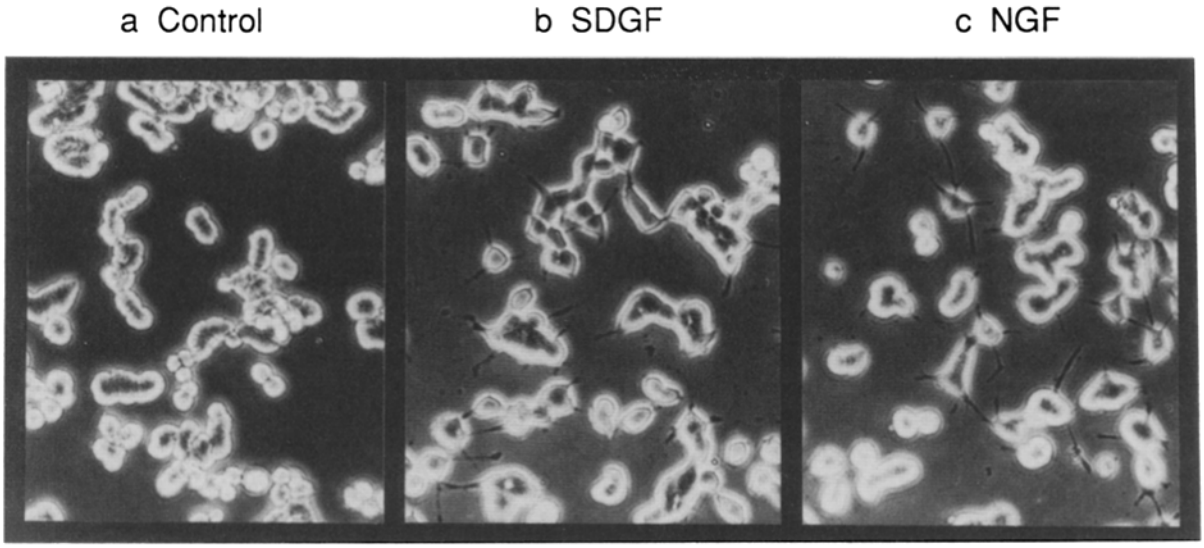

Figure 1. Morphological changes in PC12 cells treated with SDGF and NGF. PC12 cells grown on poly-L-lysine-coated tissue culture dishes in N2 medium treated with control (*a*), SDGF (10 ng/ml) (*b*), or NGF (10 ng/ml) (*c*) for 24 h. The diameter of untreated cells is 15 μm.

The concentration dependency of the effect of SDGF was measured by using GAP-43 cDNA as a probe. The optimal induction of GAP-43 mRNA was at a concentration of 10 ng/ml (300 pM), with half-maximal effects at 2.5 ng/ml (Fig. 5). The potency of SDGF was similar to that of NGF (Fig. 5). These concentrations are in close agreement with the dose-response relationship observed for the mitogenic response of SDGF on Swiss 3T3 cells, and primary cultures of astrocytes and for the survival activity on PC12 cells (Fig. 3; Kimura et al., 1990).

Effects of Dexamethasone, Cycloheximide, and MTA on Induction of GAP-43 and NGFI-A

Since SDGF induces GAP-43, transin and NGFI-A in a similar manner to NGF, we tested several agents which are known to alter the induction of these gene products to determine if there are differences between these two growth factors. Cycloheximide, a protein synthesis inhibitor, increases the induction of NGFI-A by NGF (Fig. 5 *a*; Milbrandt, 1987). Cycloheximide also increased the NGFI-A mRNA

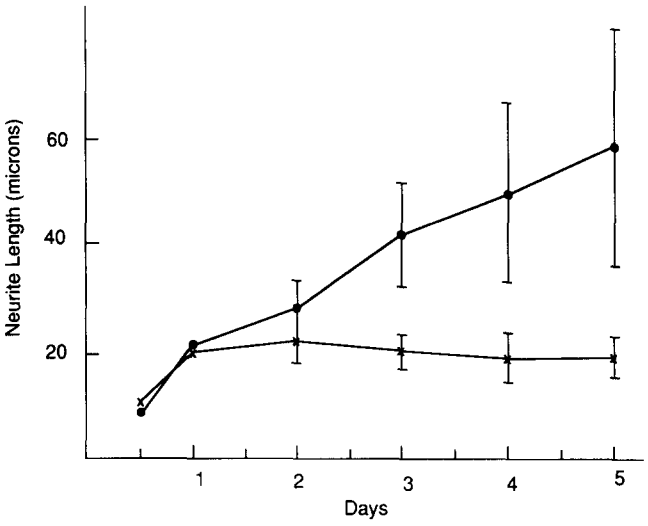

Figure 2. Rate of neurite outgrowth. Exponentially growing PC12 cells were plated on polylysine-coated tissue culture dishes in N_2 medium (Schubert et al., 1987). NGF(10 ng/ml) or SDGF(10 ng/ml) were added and neurite length determined using phase optics and an eyepiece micrometer. Over 50 cells were examined for each point and the data presented as mean neurite length plus or minus the standard error of the mean. -x-, SDGF; -●-, NGF. The cell diameter was 15 μm and control cultures had no neurites.

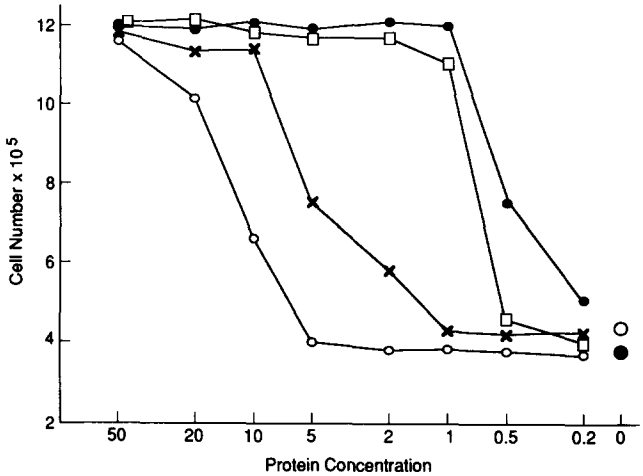

Figure 3. SDGF promotes PC12 cell survival. Exponentially dividing PC12 cells were washed three times with serum free DME and plated at 1.2 × 10^5 cells per 35-mm polylysine-coated tissue culture dish in the same medium containing varying amounts of beta-NGF and SDGF. 24 and 48 h later viable cell number was determined (Schubert et al., 1990) and this number plotted against protein concentration of each growth factor in ng per ml. (-●-) NGF, 24 h; (-□-) NGF, 48 h; (-x-) SDGF, 24 h; (-○-) SDGF, 48 h. Control (no factor) survival after 24 h (-○-) and 48 h (-●-). Variation between duplicate cultures was <10%.

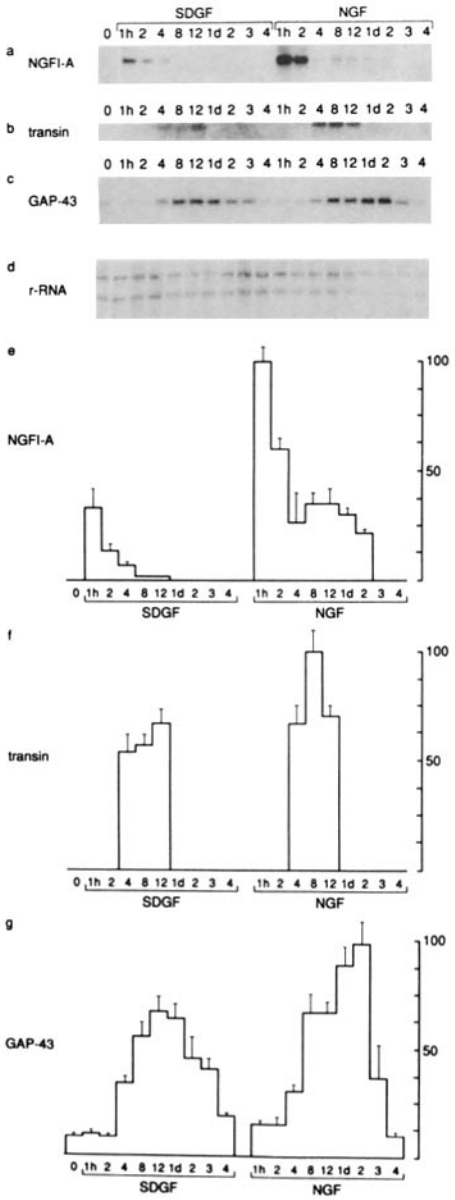

Figure 4. The time courses of NGFI-A, transin and GAP43 mRNA induction by SDGF and NGF. PC12 cells were treated with a saturating amount (8%) of serum-free conditioned medium, from 293 cells transformed with the SDGF cDNA in an expression vector (Kimura et al., 1990), or with NGF (10 ng/ml) which gave maximal responses. The cells were harvested at various times for RNA blot analysis of NGFI-A (*a*) transin (*b*) and GAP43 (*c*) mRNA levels. The same RNA blot was stripped and reprobed. The membrane was stained with methylene blue (*d*) before hybridization. Scanning laser densitometry was performed for films exposed to a membrane hybridized with probes for NGFI-A (*e*), transin (*f*), and GAP-43 (*g*). Ordinate shows the relative amount of induced mRNAs when the maximum value is given as 100. Each value represents the average of two experiments.

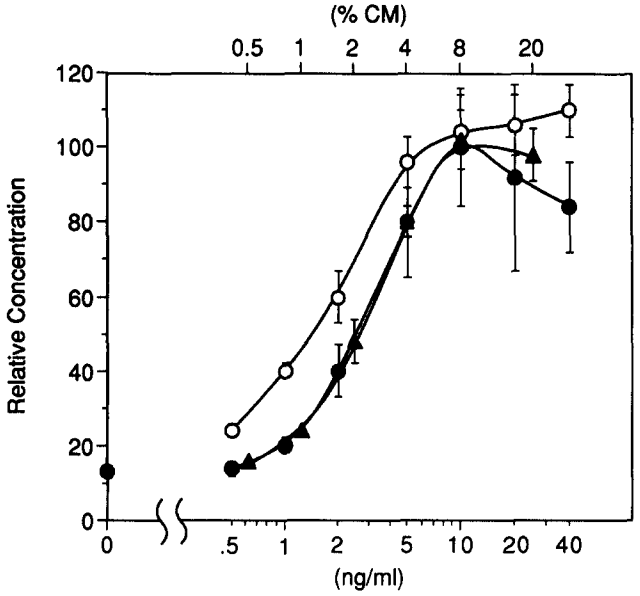

Figure 5. Dose-response curve for the induction of GAP43 mRNA in PC12 cells by SDGF and NGF. Cells were grown for 8 h in various concentrations of SDGF, NGF, and the serum-free conditioned medium of 293 cells transfected with SDGF expression plasmid (Kimura et al., 1990). Total RNAs were isolated and Northern blot analysis was performed by the method described in Materials and Methods. Scanning laser densitometry was performed on an LKB Ultroscan. Each point represents the mean of three determinations when the relative value at concentration of 10 ng/ml of SDGF is given as 100. (-●-) SDGF; (-○-) NGF; (-▲-) growth conditioned medium of 293 cells transfected with SDGF expression plasmid.

levels induced by SDGF (Fig. 6, *a* and *e*). The increase is, however, greater with SDGF than with NGF (Fig. 6, *a* and *e*). Although the control and SDGF-induced levels of GAP-43 mRNA are raised by cycloheximide, the ratios between control and induced levels are reduced (Fig. 6 *f*).

PC12 cells are derived from a rat adrenal medullary pheochromocytoma and are bipotential, becoming more neuronal with NGF and acquiring more chromaffin cell characteristics with exposure to corticosteroids (Schubert et al., 1980; Federoff et al., 1988). We therefore compared the effects of SDGF and NGF on the induction of GAP-43 and NGFI-A mRNAs in the presence of dexamethasone. Dexamethasone suppresses GAP-43 mRNA induction by these growth factors; it also inhibits neurite growth under these growth conditions. This blockade by dexamethasone was augmented by cycloheximide (Fig. 6, *c* and *f*). In contrast there was no change in NGFI-A mRNA induction, by either SDGF or NGF, in the presence of dexamethasone (Fig. 6, *a* and *e*).

5′-S-methyl adenosine (MTA), an inhibitor of phospholipid methylation, inhibits the effect of NGF on PC12 cells (Seeley et al., 1984). We therefore tested for GAP-43 and NGFI-A mRNA induction by SDGF in the presence of this agent and compared it to NGF. GAP-43 mRNA induction by both SDGF and NGF was inhibited by MTA (Fig. 6 *c* and *f*). NGFI-A induction by NGF was also inhibited by this agent, while that by SDGF was only slightly increased (Fig. 6 *a* and *e*).

Discussion

SDGF is a peptide growth factor discovered through its potent mitogenic activity for fibroblasts and glial cells (Kimura et al., 1990). In addition to its mitogenic activity, the present findings demonstrate that SDGF reproduces many of the responses previously shown to be elicited by NGF on PC12

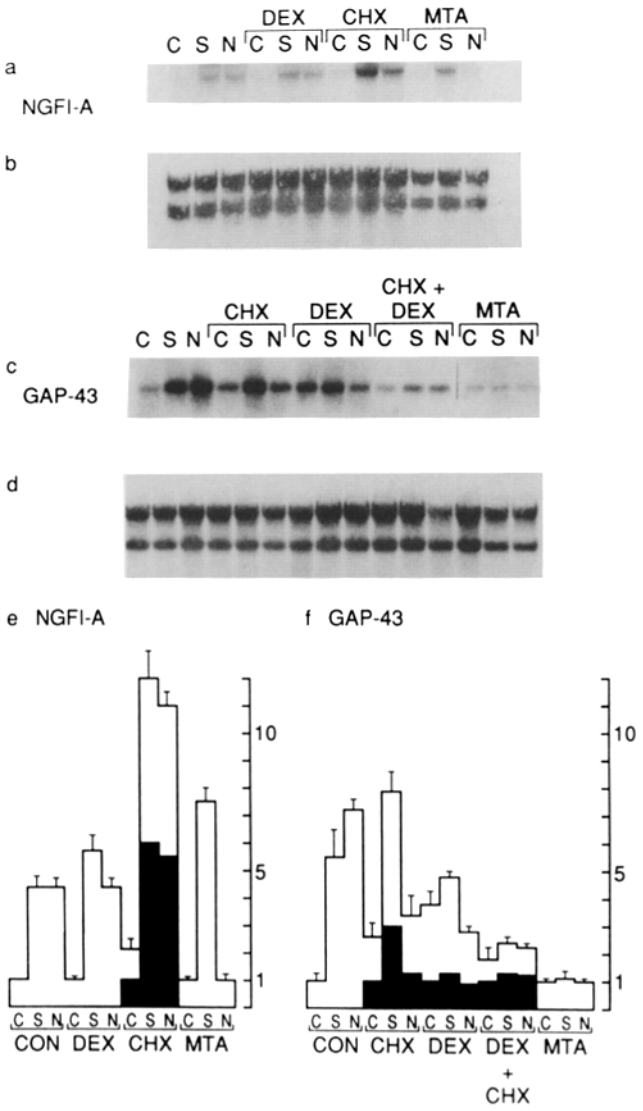

Figure 6. The effects of cycloheximide, dexamethasone, and MTA on NGFI-A and GAP43 mRNA induction by SDGF and NGF. (*a*) PC12 cells were treated with SDGF (10 ng/ml) and NGF (4 ng/ml) for 1 h. Dexamethasone (1 μM) was applied 24 h before the treatment with SDGF or NGF. Cycloheximide (1 μg/ml) and MTA (3 mM) were add simultaneously with SDGF and NGF. (*b* and *d*) Membrane filter blotted with RNAs was stained with methylene blue. (*c*) PC12 cells were treated with control (*C*), SDGF (*S*) (10 ng/ml), or NGF (*N*) (10 ng/ml) for 8 h. Applications of dexamethasone, cycloheximide, and MTA were the same as in *a*. Scanning laser densitometry *e* and *f* was performed on films of *a* and *c*. Ordinate shows the relative amount of induced mRNAs when the control value is given as 1. Shaded boxes represent the ratio to the control of each agent. In cycloheximide treated group, for example, the control value is 2.7; SDGF, 8.0; NGF, 3.5. When the control value is normalized to 1, then the value of SDGF becomes 8/2.7=2.9 and that of NGF, 3.5/2.7=1.3.

cells. Both NGF and SDGF promote neurite outgrowth in PC12 cells, and induce the mRNAs for two differentiation markers, GAP-43 and transin as well as the immediate early response gene NGFI-A, with similar time courses. There were, however, several differences between the action of the two peptides. Although the rate and extent of neurite formation induced by NGF and SDGF are the same until 24 h, outgrowth due to SDGF ceases at that time while cells in the presence of NGF continue to extend neurites for several days (Figs. 1 and 2). The induction of NGFI-A and GAP-43 by NGF also lasts longer than that by SDGF. The observed survival activity of NGF on PC12 cells may reflect this long lasting induction of these mRNAs (Fig. 3). In addition, both NGF and FGF induce the differentiation of chromaffin cells into sympathetic neurons, but only NGF can support the subsequent survival of the resultant neurons (Unsicker et al., 1978; Doupe et al., 1985; Stemple et al., 1988). SDGF might, therefore, be involved in the initial outgrowth of neuronal processes and the subsequent maintenance of cells requires the support of other growth factors such as NGF. Another difference between SDGF and NGF is that NGF induces NGFI-A mRNA to a greater extent than SDGF and this induction by NGF was suppressed completely by MTA, while that by SDGF was slightly augmented. Seeley et al. (1984) and Maher (1988) reported that another early response of PC12 cells to NGF, phosphorylation of cellular proteins, was blocked by MTA, while phosphorylation caused by EGF was somewhat enhanced by this methyl transferase inhibitor. These findings indicate that the early cellular responses to NGF and growth factors which belong to the EGF family may be controlled by distinct pathways.

Although SDGF was isolated as a mitogen (Kimura et al., 1990), its differentiation and survival effects have not been reported. Recently, several growth factors have been found to have more than one activity. Like SDGF, FGF was originally characterized as a potent mitogen (Baird and Bohlen, 1990), but it also supports the survival of embryonic rat hippocampal neurons (Walicke et al., 1986), cerebral cortical neurons (Morrison et al., 1986), cerebellar granule cells (Hatten et al., 1988), and chick parasympathetic ciliary ganglion neurons (Schubert et al., 1987; Unsicker et al., 1987). In addition, FGF potentiates neurite outgrowth in primary cultures of chromaffin cells (Stemple et al., 1988) and in PC12 cells (Togari et al., 1986; Wagner and D'Amore, 1986; Rydel and Greene, 1987; Schubert et al., 1987). Mature chromaffin cells are induced to transdifferentiate into sympathetic neurons by NGF (Unsicker et al., 1978; Doupe et al., 1985); NGF also simultaneously serves as a mitogen for these cells (Lillien and Claude, 1985). EGF, a potent mitogen for cells of ectodermal and mesodermal origin, has neurotrophic activity on subneocortical telencephalic neurons (Morrison et al., 1987). TGFalpha which apparently shares its receptor with EGF, is also a mitogen for cells of ectodermal and mesodermal origin (Schreiber et al., 1986). The TGFalpha mRNA is expressed in several areas of the brain (Kobrin et al., 1986; Wilcox and Derynck, 1988), and TGFalpha induces neurite outgrowth in PC12 cells (Zhang, 1990). Finally, activin, which was first isolated as a peptide hormone that stimulates the release of FSH from pituitary cells (Mason et al., 1985; Vale et al., 1986), causes the survival of neurogenic P19 teratoma cells and some types of nerve cells (Schubert et al., 1990). Moreover, when P19 cells are cultured on substrata of extracellular matrix protein such as laminin and fibronectin, activin is a potent mitogen (Schubert and Kimura, 1991). The above data, in conjunction with our previous finding that SDGF is expressed in embryonic brain and sciatic nerve, suggests that SDGF might be another potential candidate for a multifunctional differentiation factor in the nervous system.

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