Nerve growth factor activates Thy-1 and neurofilament gene transcription in rat PC12 cells

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The effect of nerve growth factor (NGF) on the expression of neurofilament and Thy-1 genes in rat PC12 pheochromocytoma cells was examined at both the transcriptional and post-transcriptional levels. Addition of NGF to cultured PC12 cells produced increases in mRNAs corresponding to the 68 kd neurofilament protein (NF68) and the Thy-1 glycoprotein within 24 h, with maximal effects of some 90- and 45-fold stimulation (relative to β -actin mRNA) being observed after 12 and 4 days of treatment, respectively. In addition, transcriptional run-off analyses using isolated nuclei showed that NGF treatment resulted directly in 8- and 4-fold increases in the rate of NF68 and Thy-1 gene transcription. These gene activation events were independent of overt morphological differentiation of PC12 cells occurring both under conditions permissive and non-permissive for neurite outgrowth, and once established the new molecular phenotype was dependent upon the continued presence of NGF. This is the first molecular evidence for the reversible activation of neuron-specific genes during NGF-induced differentiation in PC12 cells.

Key words: gene activation/nerve growth factor/neurofilament protein/pheochromocytoma/Thy-1

Introduction

The polypeptide hormone nerve growth factor (NGF) generates a diverse set of responses in sympathetic and certain sensory neurons including maintenance of viability, promotion of neurite outgrowth and modulation of neurotransmitter synthesis (Greene and Shooter, 1980; Thoenen and Barde, 1980). In addition, it induces the differentiation of normal and neoplastic chromaffin cells towards a neuronal phenotype as exemplified by the PC12 cell line derived from a rat pheochromocytoma (Aloe and Levi-Montalcini, 1979; Greene and Tischler, 1982). PC12 cells grown without exogenously supplied NGF do not possess neurites and closely resemble their non-tumour counterparts, adrenal chromaffin cells. When NGF is added to PC12 cultures there is a lag of ~24 h before neurite formation is first detectable after which outgrowth occurs over several days (Greene and Tischler, 1976) concurrent with enhanced electrical excitability (Dichter *et al.*, 1977), increased RNA synthesis (Gunning *et al.*, 1981) and elevation in various protein species (Greene, 1984). In particular, increases in specific cytoskeletal (Lee and Page, 1984; Greene *et al.*, 1983), cell surface (Salton *et al.*, 1983; Richter-Landsberg *et al.*, 1985) and neurotransmitter-related proteins (Rieger *et al.*, 1980; Hefti *et al.*, 1982) occur within the time frame of

morphological differentiation. A number of these so-called delayed responses to NGF which are associated with differentiation along the neuronal pathway have been shown to be dependent upon new RNA synthesis (Burstein and Greene, 1978; Greene *et al.*, 1982), and several examples of early and transient gene activation have been implicated in the inductive process leading to establishment of the neuronal phenotype (Curran and Morgan, 1985; Feinstein *et al.*, 1985; Greenberg *et al.*, 1985; Levi *et al.*, 1985). In addition, a range of acute transcription-independent responses to NGF in differentiated PC12 cells have been described including membrane ruffling (Connolly *et al.*, 1979), growth cone migration (Seeley and Green, 1983), enhanced uptake of amino acids (McGuire and Greene, 1979) and changes in protein phosphorylation (Halegona and Patrick, 1980).

Thus in PC12 cells, NGF can be shown to influence the expression of proteins at transcriptional, post-transcriptional and post-translational levels. Only in the case of the transient activation of the ornithine decarboxylase and β -actin genes, and the *c-fos* and *c-myc* proto-oncogenes have corresponding increases in mRNA levels and transcription rates been confirmed (Greenberg *et al.*, 1985; Feinstein *et al.*, 1985). It thus remains to be demonstrated at the molecular level whether NGF-induced differentiation of PC12 cells directly involves activation of phenotype-related genes and, if so, whether the induced molecular phenotype is stable.

To address these questions we have determined the level of mRNAs and transcriptional rates corresponding to the 68-kd neurofilament protein (NF68) a component of the axonal cytoskeleton, and the cell surface Thy-1 glycoprotein during NGF-induced neuronal differentiation in PC12 cells. At the protein level, both these gene products have been shown to be increased in response to NGF (Lee and Page, 1984; Richter-Landsberg *et al.*, 1985). In addition we have compared culture conditions permissive or non-permissive for neurite outgrowth and the effect of NGF withdrawal from fully differentiated cultures.

Results

In the first series of experiments, the level of NF68, Thy-1 and β -actin mRNAs were examined in control PC12 cells grown in the absence of NGF and in PC12 cells which had been exposed to NGF (10 ng/ml) over a 14-day culture period. On collagen-coated culture dishes NGF treatment results in extensive neurite outgrowth involving >95% of cells (Figure 1a,b). Under these conditions, Northern blot analyses of poly(A)⁺ RNA from control and NGF-treated cells revealed two NF68 mRNAs of 2.4 and 3.4 kb and one Thy-1 mRNA of 1.8 kb similar in size to those observed in whole rat brain (Julien *et al.*, 1985; Moriuchi *et al.*, 1983). All three RNA species were, however, significantly elevated following NGF-induced differentiation (Figure 2). In contrast, the level of β -actin mRNA was similar under both culture conditions.

To determine whether NGF-induced increases in NF68 and Thy-1 mRNAs were directly linked to the process of neurite outgrowth *per se*, cultures produced on naked tissue culture dishes

Fig. 1. Effects of NGF and culture substratum on PC12 cell morphology. Control PC12 cultures (a and c) and cultures treated for 10 days with NGF (50 ng/ml, b and d) grown on collagen-coated (a and b) or untreated (c and d) plastic tissue culture dishes. In e and f, cells treated for 14 days with NGF as in b were detached, washed and replated on collagen in the presence of either anti-NGF antibody (e) or fresh NGF (f) for a further 2 days. Cultures were photographed using phase contrast optics. Bar represents 50 μ m.

were also examined. Under these conditions, PC12 cells adhere less firmly to the culture surface with an increase in cell aggregation and virtually complete inhibition of NGF-induced neurite outgrowth (Figure 1c,d). Despite the absence of neurite outgrowth on uncoated dishes, NGF-stimulated NF68 and Thy-1 mRNA levels in a fashion similar to that observed under conditions permissive for neurite generation (results not shown).

In order to examine the time course and maximum extent of NGF-induced increases in mRNA expression, total RNA samples were prepared from a series of smaller scale cultures at various times (21 h to 18 days) following addition of a maximal dose of NGF (50 ng/ml). Northern blot analysis revealed significant increases in NF68 and Thy-1 mRNAs within 21 h following addition of NGF, the responses appearing to peak by 4-day exposure (Figure 3). In contrast, however, relative to the total RNA content of the cultures, the level of β -actin RNA gradually declined during the course of differentiation. This effect most likely

reflects the previously reported increase in total RNA content (predominantly ribosomal) in NGF-treated PC12 cells (Gunning *et al.*, 1981). Figure 4 shows the time course of NGF-induced differentiation expressed as the ratio between the hybridization intensity (as measured by scanning densitometry) of probes with the NF68 and Thy-1 mRNAs compared with that with β -actin RNA. The 3.4-kb NF68 RNA and the Thy-1 RNA were maximally elevated after 4 days treatment with NGF to 10- and 45-fold respectively relative to control cells. In contrast, the 2.4-kb NF68 RNA showed maximum stimulation in a more delayed fashion, peaking by day 12 to a level 90 times that found in untreated cultures.

In the case of the the NF68 and Thy-1 mRNAs, previous Southern blotting studies on homologous restriction enzyme fragments in the rat genome have indicated that these species are transcribed from single copy genes (Julien *et al.*, 1985; Moriuchi *et al.*, 1983). To further define the molecular mechanisms underlying

Fig. 2. Levels of NF68, Thy-1 and β -actin mRNAs in control and NGF-treated PC12 cells. Cultures were grown on collagen-coated dishes and poly(A)⁺ RNA was prepared from control cells (lane 1) and from cells treated for 14 days with NGF (lane 2). RNA samples (1 μ g) were processed for Northern blot analysis, hybridized to (A) NF68, (B) Thy-1 and (C) β -actin cDNA probes and the filters autoradiographed at -80°C in the presence of intensifying screens.

Fig. 3. Time course of NGF-induced elevation in NF68 and Thy-1 mRNAs in PC12 cells. Total RNA was prepared from PC12 cells grown on collagen-coated dishes either in the absence of NGF (lane 1) or after exposure to NGF (50 ng/ml) for 21 h (lane 2), 4 days (lane 3), 12 days (lane 4) or 18 days (lane 5). RNA samples (10 μ g) were processed for Northern blotting, hybridized to (A) NF68, (B) Thy-1 and (C) β -actin cDNA probes, and the filters autoradiographed as in Figure 2.

the increased expression of NF68 and Thy-1 mRNAs in NGF-treated PC12 cells, nuclei were isolated from control and differentiated cultures and processed for transcriptional run-off analyses (Greenberg and Ziff, 1985; Groudine *et al.*, 1981). In this assay, new RNA transcripts are not initiated but transcripts which are already initiated are faithfully elongated giving an accurate measure of the relative level of transcription at the time of cell lysis. Run-off transcripts labelled with ^{32}P were prepared using

Fig. 4. Quantitation of NGF-induced increases in NF68 and Thy-1 mRNAs. Autoradiographic bands corresponding to (A) NF68 — 3.4-kb species, (B) NF68 — 2.4-kb species and (C) Thy-1 mRNAs shown in Figure 3 were subjected to scanning densitometry and the integrated peak densities obtained presented as ratios relative to that for β -actin at each time point to yield an arbitrary measure of relative hybridization intensity.

nuclei isolated from 7×10^7 cells and hybridized to NF68 and Thy-1 cDNA inserts which had been denatured and immobilized on Genescreen membrane. Autoradiographic and subsequent scanning densitometric analyses of hybridization signals showed that 7-day treatment of PC12 cells with NGF (50 ng/ml) resulted in increases of 8- and 4-fold, respectively in the transcription rate of the NF68 and Thy-1 genes compared with control untreated cells (Figure 5).

Finally, in order to determine whether maintenance of elevated NF68 and Thy-1 mRNA levels in differentiated neuronal PC12 cells required the continued presence of NGF or represented a relatively stable phenotypic switch, cells exposed to NGF for 10 days were detached from their culture surface, washed to remove NGF and replated in the presence of either fresh growth factor or neutralizing antibody to NGF (Burststein and Greene, 1978). Under these conditions cells form large attached aggregate cultures which produce extensive neurite networks within 48 h in the presence of NGF (Figure 1f), but exhibit little process formation in the absence of the factor (Figure 1e). Total RNA was purified from PC12 cells 2 days after sub-culture and subjected

Fig. 5. Effect of NGF on transcription of NF68 and Thy-1 genes in PC12 cells. NF68 and Thy-1 cDNAs were denatured and dot-blotted onto Genescreen filters at 1:5 serial dilutions beginning with 1 μ g on the left. Filters were hybridized to 32 P-labelled run-off transcripts prepared using nuclei isolated from PC12 cells grown on collagen-coated dishes for 7 days in the absence (A) or presence (B) of 50 ng/ml NGF. Autoradiographs were produced as in Figure 2.

Fig. 6. Effect of NGF withdrawal on NF68 and Thy-1 mRNA levels in NGF-induced PC12 cells. Total RNA was prepared from control PC12 cells (1) and cells treated with NGF (50 ng/ml) for 10 days (2). In addition, NGF-treated sister cultures were suspended by mild trituration and collected by centrifugation. These cells were then reseeded in the presence of either fresh growth factor (3) or of anti-NGF antibodies (4), and RNA purified after a further 2 days incubation. RNA samples (5 μ g) were processed for Northern blotting, hybridized to (a) NF68, and (b) Thy-1 cDNA probes and autoradiography performed as in Figure 2.

to Northern blot analyses. As seen in Figure 6, withdrawal of NGF from differentiated cultures coupled with immuno-neutralization of residual growth factor resulted in the down-regulation of NF68 and Thy-1 mRNAs and their return towards control levels following withdrawal of NGF indicating the reversibility of the NGF-induced neuronal phenotype.

Discussion

NGF-induced cellular differentiation is characterized by dramatic morphological and gene expression changes. Due in part to the lack of appropriate molecular probes, the mechanism(s) by which NGF may produce altered patterns of gene expression is poorly understood. The present study shows that during NGF-induced differentiation of PC12 cells from a chromaffin cell-like to a neurite-bearing neuronal phenotype, activation of NF68 and Thy-1 genes, in addition to elevation of corresponding mRNAs occurs. These effects were found to correlate temporally with neurite outgrowth (Greene and Tischler, 1976) and with previously reported increases in NF68 and Thy-1 protein levels (Lee

and Page, 1984; Richter-Landsberg *et al.*, 1981). In addition, these phenotypic switches were found to be independent of overt morphological differentiation, i.e. neurite outgrowth, and, once established, were dependent upon the continuing presence of the initiating effector NGF. These observations are essentially compatible with the model of NGF action proposed by Greene (1984) in which induction of the neuronal phenotype the so-called 'priming' effect involves delayed transcription-dependent events, whereas expression of overt morphological differentiation requires, in addition, a set of acute transcription-independent responses to NGF (Burstin and Greene, 1978) and a substratum compatible with neurite outgrowth (Greene and Tischler, 1976).

Our observations on the reversibility of the induced NF68 and Thy-1 mRNA levels subsequent to NGF withdrawal, are further consistent with early reports that NGF effects on PC12 cells are fully reversible, with the capacity to regenerate neurites being lost and cell division recommencing 2–6 days after removal of NGF (Greene and Tischler, 1976). At the translational level, Lee and Page (1981) have reported the rapid reaccumulation of NF68 protein following replating of differentiated PC12 cells which have been divested of their neurites. These authors did not, however, examine the effect of NGF withdrawal. With respect to the Thy-1 antigen, while incorporation of [3 H]fucose into Thy-1-associated glycoprotein has been shown to remain NGF-dependent in NGF-treated cells, absolute levels of Thy-1 have not, however, been directly examined in this situation. The present results would suggest that the high levels of NF68 and Thy-1 protein expression characteristic of differentiated PC12 cells will be reversible NGF-dependent phenomena. In view, however, of the apparent transcriptional independence of the rapid NGF-induced regeneration of neurites exhibited by these cells, the involvement of translational and/or post-translational controls sensitive to other NGF-dependent metabolic events cannot be excluded.

With respect to the two NF68 RNA species detected in PC12 cells and, indeed, in rat and mouse brain, it is not yet known whether these arise as a result of splicing of the same RNA precursor, alternative polyadenylation signals or some other mechanism (Julien *et al.*, 1985; Lewis and Cowan, 1985). It is of interest, however, that during PC12 cell differentiation the increases in these two RNA species are temporally and quantitatively distinct indicating a lack of direct coordinate regulation. In mouse brain, at least from embryonic day 17 through to adulthood, these two RNA species appear to be present in approximately equimolar amounts (Lewis and Cowan, 1985). Further developmental studies are required to determine whether the differential regulation observed in PC12 cells may reflect an early embryonic switching pattern.

The ability of NGF both *in vivo* and *in vitro* to induce trans-differentiation of adrenal chromaffin cells to sympathetic neurons suggests that these cells may share a common precursor in the migrating neural crest and that environmental factors such as local NGF and corticosteroid levels may regulate this developmental decision (Landis and Patterson, 1981). Our data illustrate this phenotypic plasticity at the level of RNA and gene transcription, and it will be intriguing to determine, as is the case with normal chromaffin cells (Doupe *et al.*, 1985), whether corticosteroids antagonize induction or affect stability of the neuronal PC12 cell phenotype, as suggested by Anderson and Axel (1985). Lee and Page (1981) have indicated that hydrocortisone does not influence NF68 protein expression in PC12 cells. Recourse to a serum-free defined medium culture system may, however, be necessary to allow corticosteroid effects to be examined with certainty.

Finally, the homogeneous clonal nature of the PC12 culture system lends itself to further examination of the molecular mechanisms regulating neurofilament and Thy-1 gene expression in terms, for example, of *cis*-acting transcriptional elements (Gopal *et al.*, 1985) and DNA methylation events (Busslinger *et al.*, 1983). Investigations of this nature should help not only towards understanding the mode of action and function of NGF itself, but also towards elucidating the role of specific gene products in the induction and stability of the mature neuronal phenotype.

Materials and methods

Cell culture

PC12 cells were obtained from Lloyd Greene and grown in RPMI or Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS) and 10% horse serum (HS) as previously described (Greene and Tischler, 1976). For studies on neuronal differentiation, cells were subcultured onto either collagen-coated or untreated plastic culture dishes in medium containing 1% HS and in the presence of NGF (10–50 ng/ml) purified from mouse submaxillary gland. Cultures were given 50% media changes every 48 h. The use of reduced serum levels was found to increase significantly the long-term cell viability and total RNA yields in NGF-treated cultures but did not alter the relative levels of the mRNA species examined or their responses to NGF. For studies on NGF withdrawal, cells grown for 10 days in the presence of NGF were rinsed, detached from the culture dish by repeated pipetting and collected by centrifugation. The washed cells were then reseeded on collagen-coated dishes in the presence of either fresh NGF or of neutralizing antibodies to NGF (Collaborative Research; 1:1000 dilution).

Northern blot analysis

Isolation of total and poly(A)⁺ RNA, glyoxal-agarose gel electrophoresis and capillary blotting to Genescreen transfer membrane (New England Nuclear) was performed as previously described (Dickson *et al.*, 1986). Filter hybridizations were performed in the presence of formamide (50% v/v) and dextran sulphate (10% w/v) using cDNA probes labelled with ³²P (1–5 × 10⁸ c.p.m./μg) by a replacement synthesis method (Dickson *et al.*, 1986). Electrophoretically purified cDNA inserts serving as probes were (i) a 600-bp *Bgl*II/*Xho*I fragment from the rat NF68 cDNA p567c (Julien *et al.*, 1985), (ii) a 355-bp *Alu* fragment corresponding to bases 34–389 of the rat Thy-1 cDNA pT64 (Moriuchi *et al.*, 1983; obtained from Dr N. Barclay), and (iii) a full-length chick β-actin cDNA (Cleveland *et al.*, 1980). Filters were routinely washed for 60 min in 2 × saline sodium citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1% (w/v) SDS at 65°C followed by 0.1 × SSC, 0.1% SDS at 25°C for 60 min, and then exposed to Fuji-RX film with intensifying screens at –80°C. For quantitation of hybridization intensities, autoradiographs were examined on a Joyce-Loebl scanning densitometer and integrated density across hybridization bands calculated in arbitrary units. All densitometric readings were performed within the linear response range of the X-ray film.

Transcriptional run-off assays

Nuclei were isolated from PC12 cultures (7 × 10⁷ cells) and run-off transcripts synthesized in the presence of [³²P]UTP essentially as described by Greenberg *et al.* (1985). The ³²P-labelled RNA transcripts were then purified (Groudine *et al.*, 1981), and filter hybridization performed with NF68 and Thy-1 cDNAs (as described above) which had been denatured and dot-blotted onto Genescreen membrane (Greenberg and Ziff, 1985). Filter hybridization, autoradiography and scanning densitometric analyses were performed as described above.

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