Effect of nerve growth factor on the expression of cell cycle regulatory proteins in PC12 cells: dissection of the neurotrophic response from the anti-mitogenic response

Leo A van Grunsven1, Nathalie Billon1, Pierre Savatier2, Annie Thomas1, José Luis Urdiales1 and Brian B Rudkin1

1Differentiation and Cell Cycle Group and 2Viral Oncogenesis and Cellular Differentiation Group, Laboratoire de Biologie Moléculaire et Cellulaire, UMR 49 CNRS/Ecole Normale Supérieure de Lyon, 46, allée d’Italie, 69364 Lyon Cedex 07, France

PC12 cells treated with nerve growth factor (NGF) undergo a G1 block and differentiate. Expression of selected cell cycle regulatory proteins was studied under culture conditions which permit observation of a differentiation response independently from a mitogenic or anti-mitogenic response. The expression of all cell cycle regulatory proteins studied is modulated by NGF addition to exponentially-growing cultures in the presence of serum. While levels of most of these proteins decrease, accumulation of cyclin D1 and the cyclin-dependent kinase inhibitor p21Cip1/WAF1 is observed. Cyclin D1-associated kinase activity is inhibited, correlating with an increase in p21 protein. PC12 cells, synchronized by serum starvation, undergo morphological and functional differentiation in the presence of NGF. Neither cyclin D1 nor p21 are present in such cultures, nor is their expression upregulated by NGF, indicating that they are not required for this process. Removal of serum from differentiated PC12 cells results in loss of these proteins, but has no effect on differentiation or the non-proliferative state in presence of NGF. Together, the results indicate that cyclin D1 and p21 are not necessary for differentiation per se, nor are they required for maintenance of the differentiated state in the absence of serum.

Keywords: NGF; PC12; cyclin D1; p21Cip1/WAF1; cell cycle; differentiation

Introduction

The study on the action of nerve growth factor (NGF) was greatly facilitated by the establishment of the rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976). This cell line displays phenotypic elements characteristic of embryonal cells of the neural crest, precursor to the adrenal medulla, capable of differentiating into adrenal chromaffin-line cells (with dexamethasone) or sympathetic neuron-like cells (with NGF) (Fujita et al., 1989). NGF is the first and best characterized member of the family of neurotrophins which includes brain-derived neurotrophic factor (BDNF), NT3, NT4/5 and ciliary neurotrophic factor (Barde, 1994). Upon addition of NGF to PC12 cell cultures, these cells stop proliferating and extend neurites that can form synapses with L6 muscle cells in culture (Schubert et al., 1977).

This process is accompanied by alterations in transcription of certain genes such as ornithine decarboxylase, neuropeptide Y, MAP-2, and transforming growth factor β1 (Feinstein et al., 1985; Allen et al., 1984; Greene et al., 1983; Kim et al., 1994) as well as the proto-oncogenes c-fos, c-myc and c-jun (Curtan and Morgan, 1985; Wu et al., 1989). NGF can bind to two receptors, a low affinity neurotrophic factor receptor (LNFR), p75LNFR (Johnson et al., 1986; Radeke et al., 1987; Chao, 1994) and a higher affinity receptor, TrkA (Kaplan et al., 1991a; Barbaric, 1994) which are both found in PC12 cells. Several investigators have suggested roles for p75LNFR in vivo. Lee et al. (1992) suggested that p75LNFR might have a role in the development of the peripheral sensory nervous system. It could also have a role in regulating apoptosis in neuronal cells (Rabizadeh et al., 1993). NGF binding to the TrkA receptor results in autophosphorylation of p140src (Kaplan et al., 1991b; Klein et al., 1991), which activates several signal transduction pathways, including the Ras/Raf/MAP kinase pathway, that lead to the responses described above.

The extracellular signal-regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway is responsible for certain neurotrophic responses in PC12 cells (Frodin et al., 1994; Traverse et al., 1994; Marshall, 1995). This is confirmed by introducing oncogenic forms of Ras or Raf into PC12 cells, both which mimic the action of NGF by activating the MAP kinase pathway and stimulating neurite outgrowth in these cells (Noda et al., 1985; Wood et al., 1993). A cessation of cell proliferation is also evidenced in K-ras infected PC12 cells (Simpson et al., 1991), reflecting the anti-proliferative effect of NGF (Greene and Tischler, 1976).

The anti-mitogenic effect of NGF correlates with an accumulation of cells in a G1-like state as evidenced by flow cytometric analysis of DNA distribution (Rudkin et al., 1989) and cyclin D1 expression (van Grunsven et al., 1996). From these studies it was evident that NGF provokes a G1 phase block, thus setting the stage for investigation into the behaviour of cell cycle regulatory proteins and their corresponding kinase activities during this process. The present report addresses this aspect of NGF action in addition to asking if the modulation of the expression of cell cycle regulatory gene expression is necessary for functional differentiation of PC12 cells.
The mammalian cell cycle is regulated by an enzyme family designated the cyclin-dependent kinases (CDK) and their activating partners, the cyclins. In the G1 phase of the cell cycle, growth factor signalling can lead to the expression of G1-type cyclins which serve to activate the CDKs 2, 4 and 6. A prominent substrate for G1 CDKs is the retinoblastoma protein (RB) (Matsushime et al., 1994). Phosphorylation of RB in mid-G1 releases factors like E2F/DP1 that are necessary for entry into S-phase (Helin and Harlow, 1993). Cyclin D1/CDK4 activity is thought to be responsible for the initial phosphorylation, whereas CDK2 may be necessary for maintaining this phosphorylated state (Elledge and Harper, 1994). These different activities facilitate the passage through restriction point and entry into S-phase (for recent reviews see Draetta, 1994; Hunter and Pines, 1994; Sherr, 1994). After passing the restriction point, the cell commits itself to complete the cell cycle in a growth factor-independent manner.

Recently, inhibitors of the CDKs have been identified that can regulate the activity of the CDK/cyclin complexes (reviewed by Hunter and Pines, 1994; Elledge and Harper, 1994; Peter and Herskowitz, 1994). The members of the family of the cyclin dependent kinase inhibitors p16INK4a, p21Cip1/WAF1 (p21) and p27KIP1 are the best characterized in vitro as well as in vivo. While p16INK4a appears to play a role in negative growth control (Kamb et al., 1994), the inhibitor p21 is implicated in cell cycle arrest and the differentiation process of certain cells (Elledge and Harper, 1994).

The findings presented herein indicate that the expression of all cell cycle regulatory proteins studied is modified by NGF treatment of exponentially-growing PC12 cultures, in the presence of serum. The G1 state in which the differentiated cells are blocked, is characterized by high levels of cyclinD1/CDK4/p21 complexes and low levels of CDC2- or CDK2-associated complexes. The kinase activity corresponding to all of these complexes is decreased upon NGF treatment. Furthermore, expression of cyclin D1 and p21 do not appear to play a role in the neurotrophic response, nor the maintenance of the non-proliferative differentiated state.

**Results**

**Antimitogenic effect of NGF on exponentially growing PC12 cells**

When exponentially-growing cultures of PC12 cells are exposed to NGF, the cells stop dividing and differentiate (Figure 1). During this experimental period, levels of cyclin A, cyclin B, CDC2 and CDK2 protein expression are significantly decreased by NGF treatment (Figure 2a). Buchovich and Ziff (1994) reported that NGF had no effect on cyclin A levels in PC12 cells. This is most probably due to the fact that the cells are still proliferating, albeit at a slightly slower rate, in cultures treated with NGF using the protocol they describe. Under the conditions used in the present study a clear cessation of proliferation is routinely evidenced (cf. Figure 1). Expression of other markers indicative of a cell's proliferative status (proliferating cell nuclear antigen (PCNA)) or the G1 to S transition (hypophosphorylated RB vs hyperphosphorylated RB) were investigated. PCNA levels drop rapidly in exponentially-growing populations treated with NGF over the experimental period (Figure 2b). A gradual loss of hypophosphorylated RB was routinely observed over the first 3 to 5 days of treatment of exponentially-growing cultures, in agreement with the results of Kalman et al. (1993). In addition to this change, a major decrease in the total amount of RB protein was consistently evidenced during exposure of PC12 cultures to NGF.

**NGF treatment results in an increased cyclin D1 expression**

An accumulation of cyclin D1 protein is observed following NGF treatment of exponentially-growing PC12 cells, corresponding to a 2-5-fold increase within 2 to 5 days (Figure 2c). This reflects an accumulation of cells in the G1 phase of the cell cycle, as determined by flow cytometric analysis and immunofluorescence studies, rather than an induction of cyclin D1 expression (van Grunsven et al., 1996). The expression of the major catalytic partner of cyclin D1, CDK4, decreases during NGF treatment with a time course similar to that observed for CDK2 and CDC2 (Figure 2c). While two forms are detected in exponentially-growing cultures, only the higher band remains present in NGF treated cultures. Unfortunately, none of the tools available for detection of CDK6 or cyclin E in other cell systems worked in PC12 cells. In contrast to the other CDKs, CDK5 expression increased in PC12 cells exposed to NGF as did the neural-specific regulatory subunit p25/p35 (Figure 2d).
**CDK2- and CDC2-associated kinase activity**

The loss of CDC2 and CDK2 as well as cyclin A and B, suggested that the kinase activity relating to these complexes would decrease as well. CDC2 and CDK2, along with their corresponding cyclins, associate with the protein p13cmd to form complexes that can actively phosphorylate histone H1 (Draetta et al., 1989). Figures 3a and 3b show a typical experiment wherein the p31cmd-sepharose-associated H1 kinase activity was assayed subsequent to NGF treatment of exponentially-growing cultures. There is a clear and marked decrease in such kinase activity, in agreement with observations of Buchkovich and Ziff (1994), which follows the loss of the respective proteins, when using the protocol described herein.

**Cyclin D1-associated kinase activity**

Exposure of exponentially-growing PC12 cells to NGF results in an apparent twofold increase in the cyclin D1-associated RB kinase activity during the first 3 to 4 days, followed by an apparent decrease (Figures 3a and 3b). When expressed as a function of the relative amount of cyclin D1 actually present in the immunoprecipitated complex, an abrupt drop in RB kinase activity is evidenced (Figure 3c). This result further indicates that, although the tools for detection of CDK6 are lacking, this kinase activity (if present) is also inhibited subsequent to NGF treatment. An increase in the amount of cyclin D1 relative to CDK4 is observed in the anti-cyclin D1 immunocomplexes obtained from NGF-treated as compared to exponentially-growing cultures. While the amount of CDK4 in total cell extracts is decreasing in response to NGF, there appears to be an increase in the amount detected in the anti-cyclin D1 immunocomplexes (Figure 4), corresponding to the upper form of the protein observed in the Western blots of total cell extracts (Figure 2c). It would appear therefore, that the remaining CDK4 in the cultures is associated with cyclin D1. This suggests that the observed decrease in kinase activity could be a result of direct inhibition of the cyclin D1/CDK4 complexes.

**p21 expression**

Metabolically-labeled cells were used to look for any increase in low molecular weight proteins in complexes immunoprecipitated with antibodies against either cyclin D1 or CDK4. Figure 5a shows the accumulation of a protein having a molecular weight of approximately 21 kDa in such complexes during NGF treatment. Western analysis of the anti-cyclin

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**Figure 2** Effect of NGF on the expression of cell cycle regulatory proteins in exponentially-growing PC12 cells. PC12 cells were cultured as described in the legend to Figure 1, in the absence (Serum) or presence of NGF (Serum + NGF). At the indicated times, cells were collected and protein extracted as described in the Materials and methods section. 30 μg of total cell extract was applied to each lane, separated by SDS–PAGE, transferred from the gel to a nitrocellulose membrane and immunoblotted with antibodies to the indicated proteins using enhanced chemiluminescence as described in the Materials and methods section. Antibodies against Cyclin A: S Reed; Cyclin D1: MAb D1-72-13G; CDK4: 2CDK4 (C22), other antibodies described in the Materials and methods section.

a) Serum Serum + NGF

b) Serum Serum + NGF

c) Serum Serum + NGF

d) Serum + NGF
D1 immunoprecipitates using an anti-p21 antibody evidenced the presence of this protein (Figure 5b). The relative amount of p21 compared to cyclin D1 appears to increase in the complexes during the treatment. Accumulation of p21 was also observed in total cell extracts (Figure 5c), with the appearance of a higher mobility band. The identity of this band is not clear at this writing. Zhang et al. (1994) have identified the phosphorylation sites that cause p21 mobility shifts but also state that the phosphorylation of these sites is irrelevant to the activity of p21-associated enzymes.

Study of the neurotrophic effect of NGF on PC12 cells synchronized by serum-starvation

Serum-starved cultures of PC12 cells can be morphologically and functionally differentiated by exposure to NGF in the absence of serum much more rapidly than exponentially-growing cultures (Rudkin et al., 1989). This is a direct result of the serum starvation procedure during which the surviving cells accumulate in the late G1 phase of the cell cycle, a few hours prior to the entry into the S phase. Addition of NGF alone (i.e. in the absence of serum) does not permit the G1 to S transition whereas addition of serum does. Since these serum-starved cells are already 'blocked' in late G1 phase of the cell cycle, it is possible to study the neurotrophic response of PC12 cells to NGF separately from the anti-mitogenic response.

Cultures synchronized by serum starvation were treated with NGF in the absence or presence of serum and protein extracts were submitted to Western blotting for analysis of selected proteins. When exponentially-growing cultures are exposed to NGF in the absence of serum, they differentiate with similar kinetics as in the presence of serum (Greene, 1978). Under such

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**Figure 3** Cyclin D1- and p13<sup>Src</sup>- associated kinase activity in immune complexes of exponentially-growing PC12 cells treated with NGF. (A) At the indicated time points, extracts (500 µg of protein) were immunoprecipitated with p13<sup>Src</sup>-Sepharose (upper panel) or inactivated Sepharose 4B (upper panel; lane C), and afterwards with anti-cyclin D1 antibody (MAb D1-72-13G) (lower panel) or with control antibody (lower panel; lane C). The immunoprecipitates were assayed for kinase activity against Histone H1 and GST-RB (C-term.), respectively. Autoradiographic exposures for upper and lower panels were 20 min and 2 h. (B) Graphic representation of kinase activity as a function of time of treatment with NGF as determined by a Quantitative Phosphoimager. Cyclin D1-associated kinase activity towards GST-RB (open circles) and p13<sup>Src</sup>-associated kinase activity towards Histone H1 (closed circles). (C) Representation of the kinase activity per cyclin immunoprecipitated (Western insert of cyclin D1 immunoprecipitated with the MAb D1-72-1 3G and revealed with the same) as a function of time.
The characteristic accumulation of cyclin D1 was observed (Figure 7a; lane 2) when exponentially-growing cultures were permitted to differentiate in the presence of NGF for three days (Figure 7b). They were then treated for three more days under selected conditions. In the presence of both NGF and serum, further accumulation of cyclin D1 and p21 levels is observed (Figure 7a; lane 3) with extensive neurite outgrowth (Figure 7c). When incubated with NGF in the absence of serum, a decrease in levels of both cyclin D1 and CDK4 was observed, rather, a decrease is evident (Figure 6a, lane 1). Serum starvation results in a decrease in the expression of cyclin D1 and p21 (Figure 6a; lanes 3 and 6). The decrease of cyclin D1 protein is expected to result from the withdrawal of growth factors from the cells (Sherr, 1994). When both serum and NGF are added to cultures synchronized by serum starvation, the cells enter into the S phase and only stop once they have reached the following G1 phase (unpublished observation). This phenomenon is reflected by the accumulation of cyclin D1 and p21 (Figure 6a; lane 4) and only moderate neurite outgrowth (Figure 6d). Exposure to NGF alone does not stimulate the expression of these proteins (Figure 6a; lane 5), while the differentiation is robust (Figure 6e). Addition of serum alone, results in the appearance of both cyclin D1 and p21 (Figure 6a; lane 7) and permits the cells to proliferate without any neurite formation (Figure 6f).

During NGF-induced differentiation, an increase in the number of cells expressing cyclin D1 in the nucleus is observed by immunofluorescence (van Grunsven et al., 1996). The decrease in cyclin D1 levels elicited by Western analysis of serum-starved cells (cf. Figure 6a; lane 3) reflects the loss of cyclin D1-associated immunofluorescence in over 95% of the cells (not shown). Subsequent addition of NGF does not cause any increase in the percentage of labeled cells (not shown), in agreement with the expression of this protein, as detected by Western analysis (cf. Figure 6a; lane 5).

When serum and NGF are withdrawn from differentiated PC12 cells, they retract their neurites and die (Greene, 1978). When only serum is withdrawn, they remain differentiated whereas when only NGF is removed, the cells loose their neurites and resume proliferation. The expression of cyclin D1 and p21 was followed under each of these experimental conditions to test whether the accumulation of cyclin D1 and p21 is essential for the maintenance of the differentiated state.
D1 and p21 is observed (Figure 7a; lane 4), while the cells maintain their differentiated state (Figure 7d) indicating the coupling of their expression to the presence of mitogenic factors. In the absence of both serum and NGF, a similar decrease in these proteins is evidenced (Figure 7a; lane 5) while most cells in such cultures have lost their neurites (Figure 7e). Finally, in the presence of serum only, no decrease in cyclin D1 or p21 is observed at this time point (Figure 7a; lane 6) while the cells lose their neurites and start proliferating (Figure 7f).

**Figure 6** Effect of serum on the expression of cyclin D1 and p21. Exponentially-growing PC12 cultures were exposed to NGF in the absence of serum, or synchronized by serum starvation for 4 days as described in the Materials and methods section, then treated three more days with serum or NGF in the absence or presence of serum. At the indicated times, cells were photographed using Hoffman interference contrast microscopy and proteins were extracted and analysed according to Materials and methods. S=Serum and SS=Serum Starved. (a) Western analysis of total cell extracts with cyclin D1 Mab D1-72-13G and p21 Mab CP36. Lane 1: Exponentially-growing culture treated for 4 days with NGF in the absence of serum. Lane 2: Exponentially-growing culture. Lane 3: Culture serum-starved for 4 days (Considered as 'Day 0' for the results presented in lanes 4-7). Lane 4: Serum-starved culture treated for a further 3 days with NGF in the presence of serum. Lane 5: Serum-starved culture treated for a further 3 days with NGF in the absence of serum. Lane 6: Serum-starved culture treated for a further 3 days with medium alone in the absence of both serum and NGF. Lane 6: Serum-starved cultures treated for a further 3 days with serum alone. (b-f) Hoffman interference contrast pictures of selected cultures treated as described in (a). (b) Expo: Exponentially-growing culture. (c) 4 days SS → 3 days S: Serum-starved culture treated for a further 3 days to medium alone, without serum or NGF. (d) 4 days SS → 3 days NGF+S: Serum-starved culture exposed for a further 3 days with NGF in the presence of serum (note the attenuated neurite outgrowth). (e) 4 days SS → 3 days NGF−S: Serum-starved culture treated for a further 3 days with NGF in the absence of serum. (f) 4 days SS → 3 days + S: Serum-starved culture treated for a further 3 days with serum alone. The bar represents 10 μm.

**Figure 7** Effect of NGF and serum on the expression of cyclin D1 and p21 in differentiated PC12 cultures. Exponentially-growing PC12 cultures were permitted to differentiate in the presence of NGF for 3 days. They were then treated for a further 3 days under the specified conditions and analysed as described in the legend to Figure 6. S=Serum and d=days. (a) Western analysis of total cell extracts with cyclin D1 Mab D1-72-13G and p21 Mab CP36. Lane 1: Exponentially-growing culture. Lane 2: Exponentially-growing culture treated for 3 days with NGF in the presence of serum (Considered 'Day 0' for the results presented in lanes 3-6). Lane 3: NGF-treated cultures exposed to NGF in the presence of serum for a further 3 days (note the marked decrease in both cyclin D1 and p21). Lane 4: NGF-treated culture exposed to NGF in the absence of serum for a further 3 days (note the maintenance of both the cyclin D1 and p21 levels). (b-f) Hoffman interference contrast photographs of selected cultures analysed by Western presented in (a). (b) Day 0 = 3d NGF + S: Exponentially-growing culture treated for 3 days with NGF in the presence of serum (Considered 'Day 0' for the results presented in Figure 7a lanes 3-6). (c) 3d NGF + S → 3 d NGF + S: NGF-treated cultures exposed to NGF in the presence of serum for a further 3 days (note the increased neurite outgrowth). (d) 3d NGF + S → 3d NGF − S: NGF-treated culture exposed to NGF in the absence of serum for a further 3 days (note the maintenance of the extensive neurite outgrowth). (e) 3d NGF + S → 3d − S: NGF-treated culture exposed to medium alone, in the absence of both NGF and serum for a further 3 days (note the loss of neurities from the remaining cells). (f) 3d NGF + S → 3d + S: NGF-treated culture exposed to medium with serum alone for a further 3 days (note the loss of neurities and the increase of cell number). The bar represents 10 μm.
Discussion

The present study shows that the expression and activity of several cell cycle regulatory proteins are modulated during treatment of exponentially-growing PC12 cultures with NGF. In particular, an accumulation of cyclin D1/CDK4/p21 complexes is evidenced. The increased expression of the CDK inhibitor p21 correlates with the concomitant inhibition of the cyclin D1-associated RB-kinase activity, suggesting a role in the anti-mitogenic response to NGF in the presence of serum. It is difficult, however, to distinguish cellular responses that are specific for proliferation or differentiation when looking at exponentially-growing cell populations. Rudkin et al. (1989) illustrated that it is possible to manipulate the cells in such a way as to permit the study of either a mitogenic- or differentiation-specific response independently. This approach was used to evaluate the role of cyclin D1 and p21 in the actions of NGF on PC12 cells. Three major conclusions can be derived from this aspect of the study. First, there is a serum-dependent accumulation of cyclin D1 and p21 in PC12 cells treated with NGF. Second, while these proteins may be implicated in the NGF-dependent cell cycle arrest in the presence of serum, cyclin D1 and p21 are not necessary for the differentiation of PC12 cells per se. Third, neither protein is necessary for the maintenance of the differentiated state of PC12 cells in the absence of serum.

Evidence for a G1 block

It has been known for many years that addition of NGF to PC12 cells induces differentiation along with a concomitant cessation of growth (Greene and Tischler, 1976). At present, several observations clearly indicate that the anti-mitogenic effect of NGF on exponentially-growing PC12 cells is due to an arrest in the G1 phase: (i) the increased levels of cyclin D1 protein, observed by Western analysis, reflecting the accumulation of cyclin D1-positive cells labeled in the nucleus (this study and van Grunsven et al., 1996). D1 is expressed predominantly in G1 phase cells (Lukas et al., 1994) and its translocation into the nucleus, followed by its exclusion thereof, and/or its degradation are required for progression through G1 and S-phase (Baldis et al., 1993). (ii) The absence of the hyper-phosphorylated or 'active form' of the RB protein (Kalman et al., 1993 and this study). Phosphorylation of its hypophosphorylated or unphosphorylated forms is necessary for the 'inactivation' of RB in order to allow the G1/S transition (Helin and Hargohl, 1993). (iii) Flow cytometric analysis demonstrates that addition of NGF to exponentially-growing PC12 cells results in their accumulation in a G1-like state (Rudkin et al., 1989; van Grunsven et al., 1996).

Some studies suggest a role for cyclin D1 in the process of neuronal differentiation and/or neuronal function (Tamaru et al., 1994) while others show that myogenic differentiation is inhibited by an active cyclin D1 complex (Skapek et al., 1995). In agreement with the latter, the present study reports an increase in cyclin D1 levels upon treatment of exponentially-growing PC12 cultures with NGF in the presence of serum, while the associated kinase activity is inhibited concomitantly with an increase in the amount of cyclin dependent kinase inhibitor, p21. The expression of both of these proteins is tightly coupled to the continuous presence of serum in the culture medium, suggesting that the NGF induction of p21 may serve to simply neutralize the mitogenic stimulus afforded by serum growth factors.

p21 expression

The CDK inhibitor, p21, was first found in a complex with cyclin D1, CDK4 and PCNA (Xiong et al., 1992). In normal human fibroblasts, p21 mRNA levels fluctuate during the cell cycle while p21 protein levels stay constant (Li et al., 1994). Its expression has been implicated in p53-mediated growth arrest and apoptosis (El-Deiry et al., 1994; Dulic et al., 1994), cellular senescence (Noda et al., 1994) and quiescence (Noda et al., 1994; Li et al., 1994). It can also be triggered by multiple differentiation-inducing agents in haematopoietic and hepatoma cells (Steinman et al., 1995) and in human melanoma cells (Jiang et al., 1995). Furthermore, expression of p21 appears to be involved in muscle differentiation (Haley et al., 1995; Parker et al., 1995; Skapek et al., 1995) and terminal differentiation of keratinocytes (Missero et al., 1995). The expression of p21 during differentiation processes, appears to be independent of p53 (Steinman et al., 1994; Haley et al., 1995; Parker et al., 1995; Skapek et al., 1995; Missero et al., 1995).

NGF treatment of exponentially-growing PC12 cultures in the presence of serum results in an increase in the amount of p21. This CDK inhibitor is present in both active and inactive CDK complexes, the latter being characterized by increased amounts of p21 in the complex (Zhang et al., 1994). Accumulation of cyclin D1/CDK4/p21 complexes with a low level of RB kinase activity is observed during NGF-induced differentiation of PC12 cells. The relative amounts of cyclin D1 and p21 in the complexes appear to vary through the experimental period. A maximum of the p21/cyclin D1 ratio is reached within 3 to 5 days of treatment, correlating with the time course of the inhibition of cell proliferation. Although the absolute stoichiometry of cyclin D1 and p21 in these complexes was not determined, it is conceivable that the observed decrease in kinase activity is due to the inhibitory effect of the increased amount of p21 associated with the complex.

Accumulation in a naturally-occurring state within the G1 phase?

In the case of cyclin D1, the increased expression of this protein reflects the accumulation of cells in a state within the G1 phase where cyclin D1 is expressed – in the nucleus (van Grunsven et al., 1996). Along the same reasoning, the low levels of RB protein observed in NGF-treated PC12 cells may also be characteristic of a particular state within the normal G1 phase. Indeed, totipotent mouse embryonal stem cells exhibit a fluctuation of RB levels during the cell cycle, with a major decrease occurring during the G1 phase (Savatier et al., 1994). The low levels of cyclin A, cyclin B, CDC2, CDK2 and PCNA and the loss of CDC2- and CDK2-associated kinase activities are
consistent with this reasoning. While it is conceivable that the increased expression of p21 in the cyclin D1/CDK4 complex may be a direct effect of NGF addition in the presence of serum, it could also be characteristic of this naturally-occurring state in G1. If this were the case, then the effect of NGF would be upstream from the cyclin D1/CDK4/p21 complex. Further studies are required to elucidate this point.

Evidence for multiple pathways

In this study, we separated two distinct events as a result of NGF addition to PC12 cells: the arrest in G1 phase of the cell cycle when exponentially-growing PC12 cells are treated with NGF; and the differentiation, which can be studied by using serum-starved PC12 cells. When NGF is added to exponentially-growing cultures in the absence of serum, the continued proliferation and subsequent anti-mitogenic effect of NGF would appear to be independent of cyclin D1 and p21 since both are absent. Furthermore, neither cyclin D1 nor p21 proteins accumulate when serum-starved cells are stimulated with NGF. These results would suggest that when NGF is added to exponentially-growing cultures in the presence of serum, the observed effect on p21 may serve to neutralize the mitogenic stimulus of serum. They further suggest, that in the absence of serum, the G1 arrest could be regulated by a different mechanism not involving these proteins. Another interpretation could be that a specific target of cyclin D1/CDK4 kinase, normally phosphorylated to permit cell cycle progression, is not phosphorylated in the absence of serum, since this kinase is absent.

In the model systems reported thus far, p21 appears to play a role as a guardian of the non-proliferative state of quiescent, senescent and terminally differentiated cells (Li et al., 1994; Noda et al., 1994; Jiang et al., 1995). In differentiated PC12 cells maintained in the presence of NGF, but without serum, a decrease in the expression of cyclin D1 and p21 can be observed. Thus, in the absence of serum, neither p21 expression nor that of cyclin D1, appear to be necessary for the maintenance of the non-proliferative state of differentiated PC12 cells. Nor are they necessary for the morphological and functional differentiation induced by NGF in the absence of serum (Rudkin et al., 1989).

The present report offers characterization of the effect of NGF on selected cell cycle regulatory proteins and a possible mechanism for the anti-mitogenic effect of NGF on PC12 cells – the accumulation of p21 and concomitant inhibition of cyclinD1/CDK4 kinase activity. In apparent contradiction, the presence and activity of these proteins appears to be unessential for the differentiation process, for the anti-mitogenic effect in the absence of serum, as well as for the maintenance of the differentiated and non-proliferating state. These observations can be reconciled by the view that, in exponentially-growing cultures, the action of NGF serves in part, to neutralize the mitogenic stimulus of serum. In the absence of mitogenic signal, the cell no longer requires this response and could use other means to stop or prevent cell cycle progression.

There are other potential mechanisms for the anti-mitogenic effect of NGF than the ones described herein. The recently characterized CDK activating kinases (CAK) (Fesquet et al., 1993), and regulators thereof (Fisher and Morgan, 1994), could be interesting targets for NGF action on exponentially-growing PC12 cells. Moreover, other CDK inhibitors involved in cell cycle control and development, like the recently discovered p27^{kip} protein (Lee et al., 1995; Matsuoka et al., 1995) could also be potential mediators of NGF action in the presence of serum. The concomitant increase in expression of CDK5 and its neural-specific regulatory subunit, p25/p35, during NGF treatment, is in agreement with in vivo results of Tsai et al. (1994) showing their preferential co-localization in post-mitotic neurons of the CNS. The NGF-induced expression of these proteins in PC12 cells offers a model for clarification of their potential action in the differentiation process and, or maintenance of the differentiated state. While Lew et al. (1994) suggest that the active CDK5 may mediate neurofilament protein and tau phosphorylation, Tsai et al. (1994) state that no CDK5-associated kinase activity was detectable in control cells. Further studies should permit elucidation to the roles of these molecules in the anti-mitogenic and differentiation effects of NGF on PC12 cells. The use of the PC12 model as described, should permit a more selective dissection of signalling pathways implicated in the regulation of these processes.

Materials and methods

Cell culture

PC12 cells (obtained from Dr G Guroff, National Institutes of Health, USA) were grown at 37°C, 6% CO2, in DMEM (high glucose) with 6.1% donor horse serum, 6.1% fetal bovine serum, 10 mM HEPES, pH 7.2 (21°C), 87 µg of streptomycin and 87 U of penicillin per ml. Culture medium was changed three times per week, one during which they were split 1:8. NGF, prepared according to Bocchini and Angeletti (1969), was a generous gift of Dr G Guroff, or purchased from Quality Controlled Biologicals, USA. For induction of differentiation, NGF was added to cultures one day after reseeding, routinely at 2.5 x 10^4 cells per 162 cm² flask. The serum-starvation protocol consisted of washing the monolayers two to three times with medium without serum, then culturing them in the same (Rudkin et al., 1989). Routinely, cells were used after 3 or 4 days in the absence of serum.

Antisera

Antisera were generously provided by the following people: αCyclin A, B1 polyclonal antibodies by Dr S Reed; αCyclin A by Drs J Pines and T Hunter; αCyclin D1 MAb D1-72-13G (mouse MAb to cyclin D1) by Drs J-Y Kato and CJ Sherr (Matsushima et al., 1994); αCyclin D1 MAb Doc5 by Drs J Lukas and J Bartek (Lukas et al., 1994); αCyclin D1 (human, C-terminal domain) and αCDK4 (human, C-terminal) polyclonal antibodies from Dr F Hall; αPCNA MAb PC10 (D Lane) by S Dorrington; αCdc2 (C-terminal peptide) antisera G5 by Dr G Draetta; αMAB DC17 to Cdk5, αp35 polyclonal antibody by Dr L H Tsai (Tsai et al., 1994); αp21 MAB CP26 by Dr W Harper. Other antibodies were purchased from the following companies: MAb to Rb (PMG3-245) (Pharmingen, USA); αCDK2 (M2); αCDK4 (C22) from Santa Cruz Biotechnology (USA). All of the above worked on Western blots for detection of the respective proteins, yielding comparable results to those shown in the figures. The αCyclin D1 MAb D1-72-13G and the polyclonal αCDK4 (C22) were used for immunoprecipitation studies.
p13Sep was purified as described previously by Brizuela et al. (1987) and coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, France) according to the manufacturer’s instructions (efficiency approximately 98%). Typically, 6 mg of protein were coupled per ml of resin. In a characteristic experiment 80 μl of a 50% suspension was used to preclar 3 x 10^6 cells for cyclin D1 immunocomplex assays. The beads were then used for evaluation of Histone H1 kinase activity and analysed by Western blot as described below.

**Immunoprecipitation and protein analyses**

At the indicated times, cells were washed twice with PBS, collected in the same and quick frozen in liquid nitrogen. Frozen cell pellets were resuspended at 3 x 10^6/ml. Immunoprecipitation (IP) buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween-20). The following inhibitors were added: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), Aprotinin (5 μg/ml), 10 μM β-glycerophosphate, 0.1 mM sodium orthovanadate and 0.1 mM NaF. Cells were lysed for 1 h at 4°C with turning, after which the lysates were clarified by centrifugation at 10,000 g for 30 min. The protein content was determined using the technique of Sedmak and Grossberg (1977). After a pre-clearing of two times 2 h of 500 μg of total cell extract with 100 μl of a 50% solution of Protein A-Sepharose (PASS) (1 mg Protein A per ml resin; Pharmacia Biotech, France), the supernatants were precipitated for 2-4 h at 4°C with PAS precoated with the respective antibody then washed three times with IP buffer before analysis by Western blot.

When cells were used, cells were washed twice and incubated for 1 h in Cysteine and Methionine free medium (ICN) and dialysed serum. After cells were labeled 30 min with 30 μCi/ml PRO-MIX: L-[35S] in vitro Cell labeling mix (>1000 Ci/mmol) (Amersham, UK).

The immunoprecipitation was carried out as mentioned above, washed six times with IP buffer, denatured by boiling in polyacrylamide gel sample buffer, separated by 15% SDS-PAGE and analysed by autoradiography.

**Western blotting**

Proteins samples denatured by boiling in PAGE sample buffer were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell) using a Semi-Phor™ semi-dry transfer apparatus (Hoefer Scientific Instruments) according to manufacturer's instructions. Filters were blocked overnight at 4°C in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20) containing 3% powdered milk, incubated in the presence of the primary antibody for 1 h at 21°C (room temperature) in the same buffer containing 2% powdered milk or 2% BSA (Fraction V, protease free, Sigma). After three 10 min washes in TBST, filters are incubated with the appropriate secondary antibodies (Amersham) and analysed by means of Enhanced Chemiluminescence according to manufacturer's instructions (Amersham). For total protein analysis, 30 μg of total protein was put per well when the above method of extraction was used. Alternatively, the same amount of cells were directly lysed in sample buffer and applied on the gel. Similar results were obtained using either technique.

**Cyclin D1 immunocomplex assay**

The cyclin D1 immunocomplex assay was performed as described by Matsuinside et al. (1994). Briefly, Immunoprecipitations were carried out with the MAb D1-72-13G as mentioned above. For measuring kinase activity, precipitates were washed three times with IP buffer and twice with kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl, supplemented with the same inhibitors as in the IP buffer containing ±0.1 μg of soluble glutathione S-transferase-RB, 2.5 mM EGTA, 1 mM DTT, 10 μM β- NaN, 20 μM ATP, and 10 μCi/mmll [γ-32P] ATP (Amer 30 min at 30°C, boiled in polyacrylamide gel sample buffer, graphy of the dried slab gels and analysed by autoradiophoshoimager System GS-363 (BioRad).

**Preparation of the RB substrate**

Escherichia coli transformed with pGEX-Rb (773-928) was diluted 1:10 in LB incubation at 37°C. Bacteria were incubated for an overnight 37°C, and glutathione S-transferase RB protein was culture for 2 h at 37°C. Bacteria were briefly sonicated to the TNEN buffer (20 mM Tris pH 7.5, 0.5% NP40, 1 mM EDTA, 100 mM NaCl) supplemented, with the inhibitors mentioned in the section above. Cleared lysates were incubated with Glutathione-Agarose (Sigma, USA) for 2 h at 4°C. After two washes with TNEN buffer and two with kinase buffer containing 5 mM reduced 2 h at 4°C (Sigma). Free Glutathione was removed by separating on a

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References