Nerve Growth Factor Induces Transcription of the p21 WAF1/CIP1 and Cyclin D1 Genes in PC12 Cells by Activating the Sp1 Transcription Factor

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The PC12 pheochromocytoma cell line responds to nerve growth factor (NGF) by gradually exiting from the cell cycle and differentiating to a sympathetic neuronal phenotype. We have shown previously (Yan and Ziff, 1995) that NGF induces the expression of the p21 WAF1/CIP1/Sdi1 (p21) cyclin-dependent kinase (Cdk) inhibitor protein and the G1 phase cyclin, cyclin D1. In this report, we show that induction is at the level of transcription and that the DNA elements in both promoters that are required for NGF-specific induction are clusters of binding sites for the Sp1 transcription factor. NGF also induced a synthetic promoter with repeated Sp1 sites linked to a core promoter, and a plasmid regulated by a chimeric transactivator in which the Gal4 DNA binding domain is fused to the Sp1 transactivation domain, indicating that this transactivation domain is regulated by NGF. Epidermal growth factor, which is a weak mitogen for PC12, failed to induce any of these promoter constructs. We consider a model in which the PC12 cell cycle is arrested as p21 accumulates and attains inhibitory levels relative to Cdk/cyclin complexes. Sustained activation of p21 expression is proposed to be a distinguishing feature of the activity of NGF that contributes to PC12 growth arrest during differentiation.

Key words: NGF; cyclin D1; p21 WAF1/CIP1/Sdi1; Sp1; cell cycle; PC12

PC12 cells, a neural crest-derived pheochromocytoma cell line (Greene and Tischler, 1976), responds to the neurotrophin nerve growth factor (NGF) by withdrawing from the cell cycle, extending neurites, and changing from chromaffin-like cells to cells that resemble sympathetic neurons (Unsicker et al., 1978; Aloe and Levi, 1979; Anderson and Axel, 1986). Both NGF and a second growth factor, epidermal growth factor (EGF), activate the MAP kinase pathway via receptor tyrosine kinases (Gomez et al., 1990; Gotoh et al., 1990; Boulton et al., 1991; Gomez and Cohen, 1991) and induce early and delayed early response genes (Greenberg et al., 1986; Leonard et al., 1987; Gizang and Ziff, 1990). EGF, in contrast, is a weak mitogen (Greene and Tischler, 1976; Boonstra et al., 1986). NGF also gradually decreases the activities and protein levels of Cdc2 and Cdk2, -4, and -6 and decreases the level of cyclin B2 (Buchkovich and Ziff, 1994; Yan and Ziff, 1995). Cyclin D1 and p21 exert opposing effects on cell cycling. Cyclin D1 accelerates cell transit through the G1 phase by forming complexes with Cdk4 and Cdk6, which phosphorylate the retinoblastoma protein Rb (for review, see Sherr, 1995), blocking the function of Rb as a repressor of genes required for cell proliferation (for review, see Chen et al., 1995b; Beijersbergen and Bernards, 1996). The p21 protein inhibits the Cdk4/D1 and Cdk6/D1 complexes and thereby maintains the hypophosphorylated repressor state of Rb that blocks S phase entry. A high stoichiometric ratio of p21 to Cdk is required for kinase inhibition (Zhang et al., 1994). Thus, the relative levels of expression of p21, Cdk4/D1, and Cdk6/D1 may determine the proliferative state of the cell.

In this report we show that NGF selectively activates the p21 and cyclin D1 promoters by stimulating the transactivation domain of Sp1, a zinc finger transcription factor (Kadonaga et al., 1987) that binds adjacent to the TATA box of the p21 and cyclin D1 gene promoters. We discuss the significance of NGF stimulation of the p21 and cyclin D1 promoters for the mechanism of PC12 differentiation by NGF, including the possible role of cyclin D1, a G1 cyclin, in a program leading to growth arrest.

MATERIALS AND METHODS

Determination of cell number and neurite extension. Growth of PC12 cells was determined by counting the nuclei using a hemacytometer (Soto and Sonnenschein, 1985). Nuclei were isolated by lysing cells in lysis buffer (0.1% PBS, 0.5% Triton X-100, 2 mM MgCl2, 0.5% ethylenediaminetetraacetic acid, 0.05% ethylhexadecylammonium bromide).
The differentiation state of PC12 cells was determined by counting neurite-bearing cells with neurite length at least twice that of the cell body (Boulukos and Ziff, 1993). The level of neurite extension was calculated as the percentage of neurite-bearing cells relative to the total number of cells analyzed.

Isolation of rat cyclin D1 genomic DNA, Southern blot, and DNA sequence analysis. A rat genomic library cloned in χ DASH II vector (Stratagene, La Jolla, CA) was screened with the full-length mouse cyclin D1 cDNA (Matsushima et al., 1991). Of 1 million phage plaques screened, 7 positive plaques were isolated. Four positive phages with cyclin D1 inserts in size ranging from 20 kb to 10 kb were further characterized by Southern blot analysis. Phage DNA was purified with PEG 6000 and digested with restriction enzymes. DNA samples fractionated on 1% agarose gels were transferred to nylon membrane (Schleicher & Schuell, Keene, NH) after denaturing in 1 M NaCl, 0.5 M NaOH for 45 min. A 1.3 kb fragment or a 50 bp PstI fragment cDNA was used as the probe. Hybridization was performed at 55°C in QuikHyb Buffer (Stratagene, La Jolla, CA). A 5 kb NotI/Xbal fragment was subcloned into pBSKS. For sequencing of the 5' upstream region, the 5.0 kb of prCD1–5000 was digested with Smal. A 1660 bp fragment of prCD1–1414 was sequenced in both directions by Dr. B. Goldschmidt (Skirball Institute, New York University Medical Center) (1660 bp sequence submitted to GenBank database).

Northern blot analysis and Western blot analysis. Total RNA was prepared by the LiCl-guanidinium isothiocyanate method (Cathala et al., 1983). Samples (15 μg) of total RNA were separated by formaldehyde gel electrophoresis and transferred to nylon membrane (Schleicher & Schuell). Expression of p21 or cyclin D1 mRNA was measured by hybridization with the human p21 probe or mouse cyclin D1 probe, respectively. Probes were labeled by random priming (Boehringer Mannheim, Indianapolis, IN), and hybridization and washing were performed under standard conditions. Autoradiograms were scanned with an EPSON Expression 636 scanner. Ribosomal RNA was visualized by ethidium staining as a loading control. Western blotting has been described previously (Yan and Ziff, 1995). The Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were raised against a synthetic peptide (PEP2) corresponding to amino acid residues 520–538 of the Sp1 protein.

Constructs. The luciferase reporter plasmids containing various deletions of the p21 promoter sequence (Datto et al., 1995a,b) were gifts from Dr. Xiao-Fang Wang (Duke University, Durham, NC). Restriction sites in the rat cyclin D1 sequence were used to subclone a series of 5' upstream deletion constructs (see Fig. 2). The initial cyclin D1 construct prCD1–5000 was cloned by digesting the 5.0 kb fragment from pBSKS5.0 with BglII/NotI and inserting the resulting 1810 bp fragment upstream of the luciferase gene of the pGL2-basic reporter construct (Promega, Madison, WI). Deletions of sequences down to –450 were generated from the prCD1–1810 plasmid by removing different fragments from the 5' end using specific restriction sites followed by blunt end religation: GTI-Luc (HindIII/MluI); prCD1–1810, −980 to +227 (HindIII/Mull); prCD1–450, −450 to +227 (PmlI/Mull). The deletions prCD1–70, prCD1–10, and prCD1Δ450 were generated via PCR using specific primers with additional restriction sites for insertion into the pGLO-basic vector. G6TI-Luc and G3TI-Luc were constructed from plasmids G6T1-CAT and G3T1-CAT (gift of Dr. Robert Tjian, University of California, Berkeley) by replacing the CAT gene with the luciferase gene by digestion with KpnI/BamHI. G3T1-Luc was generated by digesting G6T1-Luc with SacI and religation. PCR was used to generate Gal4 (1–147) or Gal4 Sp1N (Gal4 1–147+Sp1N 83–621) by using pJDG or pJDG+Sp1N (provided by Thomas Gilmore, Boston University) (Sif and Gilmore, 1994) as template for DNA amplification. These PCR fragments were cloned into the HindIII/Xbal site of prCRSV expression vector to yield Re-Gal4 or Re-Gal4-Sp1N.

Cell culture and transfection of PC12 cells. PC12 cells were cultured on collagen-coated dishes. For experiments involving NGF or EGF induction, previously described procedures for PC12 cell culture and high efficiency polycationic liposome transfection were used (Yan and Ziff, 1995). All transfections included the plasmid RSV-Bgal as an internal control for transfection efficiency. When the amount of an expression vector was varied, the quantity of vector plasmid was held constant by the addition of empty vector plasmid DNA. NFG or EGF was added 12 hr after removal of the lipid-nucleic acid complex. The cells were harvested 72 hr after transfection for assay of luciferase or β-galactosidase or CAT activity as described below. Luciferase activity was corrected for differences in transfection by normalization to β-galactosidase levels as follows. The level of β-galactosidase in cells from each transfection (Gi) was measured, and the average of the β-galactosidase levels (Gav) for all of the transfections in one experiment was calculated. The luciferase light units of luciferase enzyme expressed in individual transfections (Li) was normalized to give the normalized light units (Lnorm) according to the formula:

\[ L_{\text{norm}} = \frac{L_i}{G_i G_{\text{av}}} \]

The “fold induction,” which is the number of normalized light units expressed in a growth factor-treated culture of cells divided by the number of normalized light units in the control culture, is also presented. Luciferase, β-galactosidase, and CAT assays. Luciferase and β-galactosidase assays were performed essentially as described by Yan et al. (1994). CAT assays were performed with the FAST CAT Green (deoxy) Chloramphenicol Acetyltransferase Assay system (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. The CAT enzymatic reaction was performed with a 5 hr incubation at 37°C. CAT activity was determined as the percentage acetylated chloramphenicol relative to the total of the acetylated and unacetylated chloramphenicol.

Nuclear extract preparation and electrophoretic mobility shift assays (EMSAs). PC12 nuclear extracts were prepared from unstimulated and NGF- or EGF-treated PC12 cells as described above. Nuclear extracts from PC12 cells were prepared essentially as described by Dignam et al. (1983). Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Oligodeoxynucleotides were synthesized and purified by OPERON Inc. The sequences of the wild-type or mutant Sp1 binding oligonucleotide used in these experiments were 5′-ATTGCATCGGCAGGGCCGAG-3′ and 5′-ATTGCATCGGTTGGGCGCCG-3′, respectively. Complementary oligonucleotides were annealed and labeled at their 5′ ends, using [γ-32P]ATP (3000Ci/mmol) and T4 polynucleotide kinase (Promega). Radiolabeled double-stranded oligonucleotides were purified through a Sephadex G-25 spin column. DNA-protein binding analysis was performed as follows. Each reaction containing 10 nm Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 2 μg of poly(dI-dC), and 10 μg of nuclear extracts in a total reaction volume of 10 μl was incubated at room temperature for 20 min. Then 1 ng of labeled probe (5–10 × 104 cpm) was added, and the incubation continued for 15 min. For competition studies, 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture before the addition of radiolabeled probe, and the mixture was incubated at room temperature for 20 min. Supershift assays were performed as described above, with the exception that subsequent to incubation of oligonucleotide probes with nuclear extracts, 1.0 μl of TransCruz gel supershift antibody against Sp1 protein (1.0 mg/ml) (Santa Cruz) was added to the reaction mixture and incubated for 45 min at room temperature. Mobility shift reactions were resolved on 4% non-denaturing polyacrylamide gels that were electrophoresed at 100 V at room temperature for 3–4 hr. Gels were visualized and exposed to x-ray film with an intensifying screen at –70°C. Autoradiograms were scanned with EPSON Expression 636 scanner.

RESULTS

We first established that PC12 cells differentiated under our conditions of treatment with NGF. When asynchronous PC12 cells were cultured in the presence or absence of NGF, the cells slowed division and underwent neuronal differentiation. Although the number of cells in a control culture lacking NGF continued to increase over a 14 d period, in agreement with previous reports (Greene and Tischler, 1976; Greene, 1978; Gunning et al., 1981; Burststein and Greene, 1982; Ignatius et al., 1985), by 8 d of NGF treatment the number of cells in the culture incubated with NGF nearly reached a plateau of growth, confirming that NGF inhibited cell proliferation during PC12 cell differentiation (data not shown). The first effects of NGF on growth rate were seen at 3–6 d of treatment. The PC12 cells also extended neurites, a characteristic of neuronal differentiation, only in the NGF-treated culture. Although after the first day of exposure to NGF only 10% of the cells extended neurites, by day 8 of NGF treatment almost all cells (95%) bore neurites two cell
NGF stimulates the p21 and cyclin D1 promoters through Sp1 binding sites

To confirm transcriptional regulation by NGF and to deduce the promoter elements activated by NGF, we analyzed the responses to NGF of the cloned p21 and cyclin D1 promoters when they were transiently introduced into PC12 cells in plasmids. In plasmid p21p-Luc, the 2.4 kb 5′ flanking region of the human p21 gene, which includes the p53 binding site located at −2.3 kb, drives the expression of a luciferase reporter gene. Plasmid p21Δ1.1-Luc, in which only residues −112 to −61 have been deleted, abolished the NGF response and diminished approximately twofold, and the NGF induction was reduced from 8.2-fold to 6.2-fold. This indicated that the NGF response depends on sequences between −2400 and −12; however, deletion of residues −112 and −61 in plasmid p21PSmaΔ1, a region that includes three additional Sp1 sites, abolished the NGF response (1.4-fold). This implicated the cluster of Sp1 sites in the mechanism of response to NGF. In confirmation, plasmid p21PSmaΔ2-Luc, in which only residues −112 and −62, a region that encompasses the second, third, and fourth promoter Sp1 sites, have been deleted from the −2400 nucleotide flanking region, responded only 2.6-fold NGF. These results indicate that both the NGF responsiveness and basal activity of the p21 promoter in PC12 cells were directly related to the number of Sp1 sites retained in the test plasmid from the six-site cluster located proximal to the TATA box.

To investigate NGF regulation of the cyclin D1 promoter, we cloned the 1.8 kb 5′-flanking region of the rat cyclin D1 gene (1660 bp of this sequence have been deposited in GenBank). As seen in Figure 4A, NGF stimulated this promoter in plasmid...
NGF activates the Sp1 transcription factor

To determine whether Sp1 sites were sufficient to direct an NGF response, in Figure 5A we assayed plasmids G3TI-Luc and G6TI-Luc, which contain three and six upstream Sp1 binding sites, respectively, linked to a core promoter with a synthetic TATA box plus an initiator element. These plasmids were stimulated 6.0- and 5.9-fold, respectively, by NGF but only 1.3-fold by EGF. The control plasmid GTI-Luc, which lacks Sp1 sites, was stimulated only 1.9-fold by NGF. In Figure 5B, the level of induction of G3TI-Luc increased progressively with increasing concentrations of NGF, whereas increases of EGF induced only modest changes. Taken together, these results confirmed that three Sp1 binding sites are sufficient to confer NGF-specific responsiveness on a core promoter.

We next analyzed the mechanism of stimulation by NGF. If NGF induced Sp1-dependent transcription by elevating either Sp1 protein levels or Sp1 DNA binding affinity, the induction by NGF would be reproduced by expression of exogenous Sp1 protein. To test this possibility, we determined the responses of the p21, cyclin D1, and multiple Sp1 site promoters to Sp1 protein expressed from the vector RSV-Sp1, with and without stimulation by NGF or EGF. Expression of Sp1 protein raised the basal activities of all three promoters (Fig. 6A–C), suggesting that the cellular levels of Sp1 did not fully saturate the transfected plasmids. In each case, NGF provided a further increase in the activity of the transfected promoters observed in the presence of exogenous Sp1. The extent of the induction of activity by NGF relative to the basal activity was similar in the presence or absence of exogenous Sp1. This suggested that the activity induced by NGF was over and above that provided by increasing Sp1 levels, indicating that it resulted from a mechanism other than increasing Sp1 levels or DNA binding. Western blotting and electrophoretic mobility shift experiments confirmed that NGF did not induce Sp1 protein expression or DNA binding affinity (see below). These effects of Sp1 and NGF were specific in as much as the Rous Sarcoma Virus (RSV) long terminal repeat in plasmid RSV-Luc did not respond to NGF, EGF, or Sp1 (Fig. 6D).

NGF activates the Sp1 transactivation domain

To determine whether NGF regulates the Sp1 transactivation domain, we assayed the effects of NGF on the activity of a chimeric protein, Gal4-Sp1, in which the Sp1 transactivation
domain is fused to the Gal4 DNA binding domain. In Figure 7, the reporter plasmid G5B-CAT, in which five Gal4 DNA binding sites are linked to the E1B TATA box, was stimulated 5.7-fold by NGF in the presence of the chimeric Gal4-Sp1 transactivator but only 1.1-fold in the presence of a control Gal4 DNA binding domain protein lacking the Sp1 transactivation domain. In neither case did the reporter respond significantly to EGF. This experiment demonstrated that the transactivation domain of Sp1 is sufficient for NGF stimulation.

Sp1 is one member of a family of factors that binds to the Sp1 DNA site. This includes Sp2, Sp3, and Sp4 as well as Sp1 itself (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Hagen et al., 1995). To determine whether NGF induces transcription by altering the proteins that bind to Sp1 sites, we compared protein–DNA complexes formed between an Sp1 site oligonucleotide and nuclear extracts of PC12 cells treated with NGF at 3 d of stimulation, a time when NGF has induced the cyclin D1 and p21 promoter activities and protein levels (Yan and Ziff, 1995), we observed a series of complexes (A–F) shown in Figure 8A. Similar complexes were also observed with extracts from unstimulated cells (lanes 1–3) and with cells treated with NGF (lanes 4–6). Each complex was specifically competed by an unlabeled wild-type Sp1 site oligonucleotide but not by a mutant. Complex A is the most prominent complex formed by extracts from cells treated for 3 d with NGF, as well as by extracts from control cells. We investigated the protein components of this complex further. In Figure 8B, complex A was supershifted by a polyclonal antibody specific for the Sp1 protein but not by a control anti-Rb protein antibody (lanes 1–3). This indicated that complex A consists of Sp1 protein bound to the Sp1 site oligonucleotide. Complex A comigrated with a complex formed by recombinant human Sp1 protein (lane 4), suggesting that Sp1 was the sole protein component in complex A. It also comigrated with a complex formed by HeLa cell extract with the Sp1 site oligonucleotide that was sensitive to the Sp1 antibody but not the RB antibody. We also assayed for changes in Sp1 protein levels induced by NGF, using Western analysis. In Figure 8C, after 1 and 3 d of NGF treatment, or as controls, after incubation with EGF or without any growth factor, PC12 cells contained comparable levels of Sp1 as revealed by Western blotting with anti-Sp1 antibody (lanes 1–6). This confirmed that treatment with NGF did not
alter Sp1 levels. We conclude that Sp1 is the major protein in extracts from control and 3 d NGF-treated cells that binds to Sp1 sites.

**DISCUSSION**

We show that NGF induces the transcription of two cell cycle regulatory genes, the p21 gene and the cyclin D1 gene. The induction is specific for NGF in as much as EGF, a weak mitogen, failed to induce. The induction of the p21 gene has been studied most extensively in the case of p53-induced growth arrest (El-Deiry et al., 1993; Dulic et al., 1994). The p53 tumor suppressor protein transactivates the p21 promoter through a site ~2000 residues distal to the transcription start (El-Deiry et al., 1993). NGF is capable of inducing p21 inde-
independently of the p53 pathway, because deletion of the p53 binding site did not impair induction. Other examples of p53 independent control of p21 have been described (Jiang et al., 1994; Steinman et al., 1994; Datto et al., 1995b; Macleod et al., 1995; Parker et al., 1995; Akagi et al., 1996; Li et al., 1996; Y. Liu et al., 1996). The p53 protein, however, may have other roles in the mechanism of NGF-induced differentiation of PC12 cells (Eizenberg et al., 1996). The cyclin D1 gene is expressed in many cells in response to mitogens and can enable cells to cross a G1 restriction point (for review, see Sherr, 1995).

NGF activates the p21 and cyclin D1 promoters via Sp1

We show that NGF activates transcription via Sp1, a transcription factor that contains a zinc finger DNA binding domain and a bipartite transactivation domain consisting of glutamine-rich regions (Courey and Tjian, 1988; Courey et al., 1989) and that interacts with two TAF proteins, dTAFII110 (Hoey et al., 1993; Gill et al., 1994) and hTAFII55 (Chiang and Roeder, 1995). Although Sp1 is linked to housekeeping gene expression, this gene class is not well defined (Datto et al., 1995b). The persistent activation (for days) of the p21 and cyclin D1 genes by NGF may place these genes in the “housekeeping” category. Sp1 also contributes to the regulation of transcription of a number of neuron-specific genes (Elder et al., 1992; Hahn et al., 1992; Chin et al., 1994; Faraonio et al., 1994; Kallunki et al., 1995; Reeben et al., 1995; Ryabinin et al., 1995; Cibelli et al., 1996).

Several experiments confirm that Sp1 is activated by NGF. Deletion of the Sp1 clusters eliminated induction by NGF. Also,
NGF activated a promoter composed of multiple Sp1 binding sites linked to a promoter core. NGF also stimulated a Gal4-Sp1 transactivation domain chimera, indicating that NGF augments the function of the Sp1 transactivation domain. Sp1 is likely to be the major factor activated in PC12 cells, in as much as Sp1 is the major protein in nuclear extracts from the NGF-treated cells that forms a complex with an Sp1 site oligonucleotide. Sp3, however, induces the p21 promoter during keratinocyte differentiation (Prowse et al., 1997). The Sp1 site cluster functions in p21 gene induction in U937 cells by okadaic acid and phorbol esters through the same Sp1 site region as studied here (Zeng et al., 1997). Although the hypophosphorylated form of Rb can activate Sp1 function (Kim et al., 1992; Udvadia et al., 1995), the Sp1-Sp3 complex may not be involved in the control of p21 by NGF. Sp1 may now be the major Sp1-binding protein activated during NGF treatment, but Sp1 activity is likely to be modulated by other agents.

Consequences of NGF regulation of the p21 and cyclin D1 promoters

Cyclin D1 is expressed in many cell types as an early response to growth factors and mitogens, and its aberrant overexpression can be oncogenic rather than growth inhibiting (Jiang et al., 1992, 1993; Juan et al., 1996; Wang et al., 1996). In fibroblasts, elevation of cyclin D1 protein can decrease the length of the G1 phase and speed cell entry into the S phase (Matsushima et al., 1992; Baldin et al., 1993). Therefore, at first consideration, induction of cyclin D1 by NGF during growth arrest seems to be a paradox. Although the current studies do not directly investigate the functions of the p21 and cyclin D1 proteins in PC12, this paradox is partially resolved by reports that...
NGF can be a mitogen during the initial stage of NGF action (Burstein and Greene, 1982). PC12 undergoes one or more rounds of DNA synthesis after NGF treatment (Rudkin et al., 1989) and begins to withdraw from the cell cycle on the third day of NGF stimulation. Cyclin D1 may contribute to PC12 proliferation during the initial stage of NGF treatment.

The arrest of PC12 growth coincides with a later stage of NGF treatment, a time when Cdk activity is inhibited and Cdk protein levels decline (Yan and Ziff, 1995). The decline in Cdk2 activity takes place between 1 and 3 d of treatment, with Cdk2 activity declining more slowly (Buchkovich and Ziff, 1994; Dobashi et al., 1995; Yan and Ziff, 1995). Cdk protein levels also decline, a change that takes place for Cdc2 and Cdk2 between 3 and 10 d of treatment. Cdk6 declines at 6 d of NGF treatment, and Cdk4 declines at 8–10 d. Cdk inhibition by p21 and decline in Cdk protein provide a basis for a barrier to PC12 proliferation.

The p21 protein forms a physical association with Cdk/cyclin complexes (Peter and Herskowitz, 1994; Waga et al., 1994) that inhibits Cdk4 and Cdk6 (Bates et al., 1994) and Cdk2 (Harper et al., 1995). Indeed, overexpression of Cdk2 can block PC12 differentiation by NGF (Dobashi et al., 1995). The p21 protein also inhibits the PCNA protein, an essential DNA replication protein (R. Li et al., 1994; Waga et al., 1994; Chen et al., 1995a; Luo et al., 1995). Cdk/cyclin/p21 complexes may exist in both active and inactive forms and are inhibited only when p21 reaches a high stoichiometric ratio (Zhang et al., 1994). The observed delay in the arrest of cycling may reflect a requirement for accumulation of p21 at high, inhibitory levels. The decline in Cdk protein may consolidate the block imposed by p21.

In particular circumstances, cyclin D1 may inhibit proliferation and be compatible with the postmitotic state. Acute expression of cyclin D1 can block fibroblast cycling through interaction with proliferating cell nuclear antigen (Pagano et al., 1994). Senescent fibroblasts contain elevated levels of cyclin D1 in complexes with an inactive, dephosphorylated form of Cdk2 (Dulic et al., 1993). Cyclin D3 is found in differentiating postmitotic myotubes in complexes with inactive forms of Cdk2 and Cdk4 (Kiess et al., 1995; Rao and Kohtz, 1995). Significantly, transient expression of cyclin D1 in 6–24 cells, a derivative of PC12 cells that overexpresses the trk-A receptor for NGF, decreased the population of S phase cells (Yan and Ziff, 1995), and cyclin D1 may contribute to the antimitotic effects of NGF in PC12 cells in the presence of serum (van Grunsven et al., 1996a). Cyclin D1 is expressed in differentiating cells in the developing CNS (Sicsinski et al., 1995) and in mature brain (Tamaru et al., 1993), including cells of the external granular layer (EGL) of the cerebellum (Shambaugh et al., 1996). Postmitotic cells of the EGL also express cyclin D2 external granular layer (EGL) of the cerebellum (Shambaugh et al., 1996). Postmitotic cells of the EGL also express cyclin D2 external granular layer (EGL) of the cerebellum (Shambaugh et al., 1996). Postmitotic cells of the EGL also express cyclin D2 external granular layer (EGL) of the cerebellum (Shambaugh et al., 1996).

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