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Nerve Growth Factor Regulates the Expression and Activity of $p33^{cdk2}$ and $p34^{cdc2}$ Kinases in PC12 Pheochromocytoma Cells

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In the absence of serum, nerve growth factor (NGF) promotes the survival and differentiation of the PC12 pheochromocytoma cell line. In the presence of serum, NGF acts primarily as a differentiation factor and negative regulator of cell cycling. To investigate NGF control of cell cycling, we have analyzed the regulation of cyclin dependent kinases during PC12 cell differentiation. NGF treatment leads to a reduction in the steady-state protein levels of $p33^{cdk2}$ and $p34^{cdc2}$, two key regulators of cell cycle progression. The decrease in $p33^{cdk2}$ and $p34^{cdc2}$ coincides with a decrease in the enzymatic activity of cyclinA-$p34^{cdc2}$, cyclinB-$p34^{cdc2}$, cyclinE-$p33^{cdk2}$, and cyclinA-$p33^{cdk2}$ kinases. The decline in $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity in response to NGF is accelerated in cells that overexpress the $p140^{trk}$ NGF receptor, suggesting that the timing of the down- regulation is dependent on the level of $p140^{trk}$ and the strength of the NGF signal. The level of cyclin A, a regulatory subunit of $p33^{cdk2}$ and $p34^{cdc2}$, is relatively constant during PC12 differentiation. Nevertheless, the DNA binding activity of the cyclinA-associated transcription factor E2F/DP decreases. Thus, NGF down-regulates the activity of cyclin dependent kinases and cyclin-transcription factor complexes during PC12 differentiation.

INTRODUCTION

An essential component of the differentiation and survival of neurons is the arrest of cell cycling. Terminally differentiated neurons of vertebrates are generally arrested in a postmitotic state with a 2n complement of DNA, although a few exceptions have been reported in which neurons arrest proliferation with a ≥4n DNA content (Lentz and Lapham, 1969; Bregnard *et al.*, 1977). In rodents, several key activators of cell cycle controls are down-regulated during the neurogenesis and differentiation of cells of the central nervous system (CNS) and peripheral nervous system (PNS) (Hayes *et al.*, 1991; Okano *et al.*, 1993; Freeman *et al.*, 1994). The down-regulation of these activators of cell cycling is most likely responsible for the postmitotic arrest of the differentiated cells. It is important to understand the mechanism(s) by which cell cycle controls are regulated during differentiation, because growing evidence suggests that the reactivation of cell cycle controls in terminally differentiated neurons leads to cell death (Al-Ubaidi *et al.*, 1992; Clarke *et al.*, 1992; Feddersen *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Hammang *et al.*, 1993) and may be the basis for neurodegenerative disease (Heintz, 1993).

The differentiation and survival of CNS and PNS neurons is influenced by neurotrophic factors. The neurotrophic factors include the neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], NT3, NT4/5), ciliary neurotrophic factor, and the fibroblast growth factors (Wagner, 1991; Hefti *et al.*, 1992). These factors display distinct temporal and spatial patterns of expression in the developing nervous system, promoting the survival and differentiation of overlapping populations of neurons. The best characterized neurotrophin is NGF. NGF is required for the differentiation and survival of sympathetic and sensory neurons of the PNS (Levi-Montalcini, 1987; Crowley *et al.*, 1994). NGF also promotes the differentiation and survival of the rat pheochromocytoma cell line PC12 into cells resembling sympathetic neurons (Greene and Tischler, 1976). NGF treatment of PC12 cells leads to transcriptionally me-

diated events, including the extension of neurites, synthesis of neurotransmitters, and the development of electrical excitability (Halegoua *et al.*, 1991).

PC12 cells have been instrumental in elucidating the NGF biochemical signaling pathway. NGF binds to two transmembrane proteins, $p140^{trk}$ and p75, on the cell surface (reviewed in Barbacid, 1993). p75 may play an important role in recruiting or presenting NGF to $p140^{trk}$, a member of the tyrosine kinase receptor family (Barbacid, 1993). The binding of NGF ligand to $p140^{trk}$ leads to the tyrosine phosphorylation of $p140^{trk}$ and the activation of its intrinsic kinase activity (Kaplan *et al.*, 1991; Klein *et al.*, 1991). These events initiate a signaling cascade involving the phosphorylation of intracellular proteins on tyrosine residues (Maher, 1988). $p140^{trk}$ physically associates with several intracellular substrates, including phospholipase C-γ1 (PLC-γ1), phosphatidylinositol 3-kinase, Erk1, and SHC (Vetter *et al.*, 1991; Loeb *et al.*, 1992; Soltoff *et al.*, 1992; Obermeier *et al.*, 1993; Stephens *et al.*, 1994). Some of these $p140^{trk}$-associated proteins possess catalytic functions, whereas others serve as adaptors to link the receptor with downstream enzymes. Studies using dominant inhibitory ras mutants indicate that $p21^{ras}$, as well as several cytoplasmic serine/threonine kinases, function downstream of $p140^{trk}$ and PLC-γ1 (Szeberenyi *et al.*, 1990; Robbins *et al.*, 1992; Thomas *et al.*, 1992; Troppmair *et al.*, 1992; Wood *et al.*, 1992). The NGF signal relayed by the $p140^{trk}$ receptor, $p140^{trk}$-associated proteins, and the cytoplasmic kinases eventually reaches the nucleus and initiates a cascade of gene expression (reviewed in Halegoua *et al.*, 1991).

Although NGF induces the differentiation of PC12 cells, epidermal growth factor (EGF) causes PC12 cells to proliferate (Huff and Guroff, 1979). Thus, although NGF and EGF activate several of the same receptor-linked enzymes, cytoplasmic kinases, and nuclear transcription factors, they have different effects on the proliferative rates and morphology of PC12 cells. The ability of NGF, but not EGF, to induce differentiation may be related to the ability of NGF to elicit a prolonged activation of the $p140^{trk}$ signaling pathway (Qui and Green, 1992) and to activate signaling molecules unique to the NGF pathway (Halegoua *et al.*, 1991; Rabin *et al.*, 1993).

The role of NGF as a differentiation factor is likely to be linked to its activity as a regulator of cell cycling. NGF treatment of PC12 cells leads to a decrease in proliferation rates, a decrease in DNA synthesis, and the accumulation of the hypophosphorylated form of the retinoblastoma protein (Greene and Tischler, 1976; Gunning *et al.*, 1981; Ignatius *et al.*, 1985; Kalman *et al.*, 1993). These effects of NGF have been interpreted as a proliferative arrest and removal of cells from the cell cycle (Greene and Tischler, 1976). However, it has been shown that NGF treatment of PC12 cells does not completely block DNA synthesis (Gunning *et al.*, 1981; Ignatius *et al.*, 1985). There is conflicting data as to whether NGF leads to an accumulation of PC12 cells in a G0/G1 (2n) phase (Ignatius *et al.*, 1985; Rudkin *et al.*, 1989) (see DISCUSSION).

In our studies we were interested in determining if NGF regulates cyclin-dependent kinases (cdks). Several cdks are required in the cells of higher eukaryotes for progression through the cell cycle. In this paper, we have investigated specifically the regulation of $p33^{cdk2}$ and $p34^{cdc2}$ by NGF.

$p33^{cdk2}$ is the catalytic subunit of a kinase that is required for cells to progress into the DNA synthesis phase (S phase) of the cell cycle. The microinjection of cdk2 antibodies (Pagano *et al.*, 1993; Tsai *et al.*, 1993) or the expression of dominant inhibitory mutants of the kinase (van den Heuvel and Harlow, 1993) blocks DNA synthesis. The binding of A- and E-type cyclins activates $p33^{cdk2}$ kinase activity; therefore, these cyclins can be regarded as positively acting regulatory subunits. D-type cyclins also associate with $p33^{cdk2}$, but it remains to be determined if cyclinD-$p33^{cdk2}$ complexes possess kinase activity in mammalian cells. Both cyclin binding and the phosphorylation of $p33^{cdk2}$ by a cdk-activating kinase(s) are required to fully activate $p33^{cdk2}$ enzymatic activity (Connell-Crowley *et al.*, 1993; Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). The activities of $p33^{cdk2}$ kinases are negatively regulated by the binding of inhibitory proteins (Gu *et al.*, 1993; Gyuris *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Polyak *et al.*, 1994).

Like the $p33^{cdk2}$ catalytic subunit, the $p34^{cdc2}$ catalytic subunit is regulated by cyclin binding, phosphorylation, and its association with other proteins (Clarke and Karsenti, 1991; Norbury and Nurse, 1992). $p34^{cdc2}$ forms complexes with A- and B-type cyclins during the proliferative phases of the cell cycle, that is S, G2, and M phases. Whereas, $p33^{cdk2}$ is required for progression into S phase in multicellular eukaryotes and $p34^{cdc2}$ is required for progression into M phase (Riabowal *et al.*, 1989; van den Heuvel and Harlow, 1993).

The cyclin-$p33^{cdk2}$ kinases link cell cycle events and transcriptional control; thus we have also investigated the effect of NGF treatment on cdk/transcription factor complexes in PC12 cells. During specific phases of the cell cycle, cyclinA-$p33^{cdk2}$ and cyclinE-$p33^{cdk2}$ kinases associate with transcription factors of the E2F/DP family (Devoto *et al.*, 1992; Lees *et al.*, 1992). The DNA binding sites for the heterodimeric E2F/DP transcription factors have been identified in the promoters of several cellular genes that are required for cell cycle progression, in particular for progression through S phase (reviewed in Nevins, 1992; La Thangue, 1994).

In this study, we have asked the following questions. Does NGF regulate the kinase activity of the $p33^{cdk2}$ and/or $p34^{cdc2}$ cataytic subunits? If so, which specific cyclin-cdk complexes are regulated? Is the expression of the $p33^{cdk2}$ and $p34^{cdc2}$ kinases affected by NGF

treatment? Are the E2F/DP transcription factor complexes regulated during PC12 cell differentiation? Finally, what are the effects of NGF on PC12 cell cycle progression?

MATERIALS AND METHODS

Cell Culture and Labeling

PC12 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% calf serum, 5% horse serum (heat inactivated, Hyclone, Logan, UT), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C, 10% CO_2. For labeling experiments, ~5×10^6 cells were plated per 10-cm tissue culture plate and allowed to adhere for several hours. Cells were washed with labeling media, either methionine-free DMEM (GIBCO) or phosphate-free Eagle's minimum essential medium (GIBCO), supplemented with 10% dialyzed calf serum, 5% dialyzed horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Two milliliters of labeling media were added to each plate along with 1.0 mCi of ^{35}S-EXPRESS Label (New England Nuclear, Boston, MA) or 1.0 mCi of ^{32}P-orthophosphate (New England Nuclear). The cells were labeled for 4–6 h (Figure 1) or 12 h (Figure 6A).

For time course experiments, PC12 cells were plated on collagen-coated (Vitrogen 100, Celtrix, Santa Clara, CA) tissue culture plates. The following numbers of cells were plated per 10-cm plate on the indicated days: 4×10^5 on day −10; 8×10^5 on day −8; 16×10^5 on day −6; 32×10^5 on day −4; 64×10^5 on day −2. Because plating the cells has been shown to transiently affect their cell cycle distribution (Ignatius *et al.*, 1985), the cells were plated 2 d before the addition of NGF. For the first addition of NGF (2.5S, Harlan Bioproducts for Science, Madison, WI), 50% of the media was replaced with media containing 100 ng/ml NGF to give a final concentration of 50 ng/ml. Every 2 d thereafter, the NGF was replenished by replacing 50% of the media with media containing 50 ng/ml NGF (Gunning *et al.*, 1981). Control cultures were plated and fed in an identical manner except for the ommission of NGF. The cells cultured in the absence or presence of NGF were inspected for neurite outgrowth. In all experiments, neurite outgrowth was apparent in the NGF-treated cells but not in the control cultures. Cells were harvested on day 0.

Cells overexpressing p140^{trk} (6–24 clonal line) (Hempstead *et al.*, 1992) and the parental PC cells were routinely cultured in DMEM supplemented with 5% calf serum, 10% horse serum (heat inactivated, GIBCO), 200 μg/ml geneticin (G418), 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at 60–70% confluencey ~12 h before the start of the experiment. At 0, 6, and 18 h before cell lysis, NGF (50 ng/ml) was added without the addition of fresh media.

PC12 cells that had been stably transfected with the adenovirus E1A gene (gE1a-PC12 cells) (Boulukos and Ziff, 1993) were cultured in DMEM supplemented with 10% calf serum, 5% horse serum (heat inactivated, Hyclone), 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Determining Cell Number

The method of Soto and Sonnenschein (1985) was used to overcome the problem of counting aggregated cells. Nuclei were isolated by mixing 100 μl of cells in DMEM and 900 μl of Lysing Solution (0.1% phosphate-buffered saline, 0.5% Triton X-100, 2 mM $MgCl_2$, 0.5% ethylhexadecyldimethyl ammonium bromide). The nuclei were counted using a hemacytometer.

Cell Lysis and Measurement of Protein Concentration

Cells were harvested and lysed by adding 1 ml of ice-cold Lysis Buffer (50 mM *N*-hydroxyethylpiperazine-*N*′-ethanesulfonic acid [HEPES], pH 7.0, 250 mM NaCl, 0.1% NP40, 10 mM sodium fluoride, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 5 mM dithiothreitol, 1 μg/ml pepstatin) to each 10-cm plate. After 30 min on ice, the lysed cells were removed from the plates by forceful pipetting. Insoluble cellular components were cleared from the lysates by centrifugation. Protein concentrations were determined using Bio-Rad (Richmond, CA) Protein Concentration Reagent. Samples were adjusted to equivalent volumes and protein concentration by the addition of Lysis Buffer.

Antibodies

cdc2 sera were raised against a peptide (CDNQIKKM, Cambridge Research Biochemicals, Northwick, Cheshire, UK) (Draetta and Beach, 1988) corresponding to the carboxy terminus of p34^{cdc2}. The peptide was coupled to keyhole limpet hemocyanin (KLH). The antigen injections and sera collection were performed at Pocono Rabbit Farm and Laboratory (Canadensis, PA). New Zealand White rabbits were injected with 200 μg peptide in the popliteal lymph nodes for the initial injection and 100 μg intradermally 2 and 4 wk later. Monthly booster injections of 50 μg were administered subcutaneously. The sera were analyzed by immunoprecipitation and immunoblotting for reactivity with p34^{cdc2}. The peptide against which the sera was raised specifically blocked the detection of a 34-kDa protein in immunoprecipitations and immunoblots. Serum obtained 8 mo after the initial injection was used in this study (rabbit 7845; sera 10–12–90).

p107 sera were raised against a peptide (CKRLQDVVSERANH, Multiple Peptide Systems, San Diego, CA) corresponding to the carboxy terminus of p107. The peptide was coupled to KLH. The antigen injections and sera collection were carried out as described above. Serum from multiple rabbits were tested for reactivity with p107 by immunoprecipitation. Sera obtained 6 wk after the initial injection was used in this study (rabbit 10395, sera 4–23–93; Figure 7A).

Purifed cdk2 polyclonal antibodies raised against a peptide corresponding to the carboxy terminus of p33^{cdk2} were purchased from Upstate Biotechnology (Lake Placid, NY). The cyclin A sera, cyclin B sera, cyclin E sera, p107 monoclonal (clone SD9), and SV40 TAg monoclonal (clone PAb416) have been described elsewhere (Harlow *et al.*, 1981; Pines and Hunter, 1989, 1990; Dulic *et al.*, 1992; Dyson *et al.*, 1993). The cyclin A and cyclin B1 sera were generously supplied by J. Pines and T. Hunter. Cyclin E sera was kindly provided by A. Koff, E. Firpo, and J. Roberts. The p107 and SV40 T antigen monoclonals were generously supplied by N. Dyson and E. Harlow. Monoclonal antibodies specific for the retinoblastoma protein (G3–245) were purchased from Pharmingen (San Diego, CA).

Immunoprecipitation and Immunoblotting

Immunoprecipitations and immunoblotting were carried out essentially as described by Harlow and Lane (1988). For immunoprecipitations, lysates were used immediately and were not frozen as this resulted in decreased kinase activity. Lysates were cleared of protein A-Sepharose (PAS)-binding proteins by incubation with 100 μl of 10% PAS (wet bead volume/buffer volume) for 1 h with rocking at 4°C. PAS was removed by centrifugation, and appropriate antibodies were added for 1 h on ice. Saturating amounts of each antibody were used, such that the addition of more antibody would not increase the signal. The immune complexes were collected by rocking with 75 μl of 10% PAS for 45 min at 4°C. Samples were washed three times in Lysis Buffer. Kinase samples were processed further as described below. Laemmli sample buffer was added.

For preclearing extracts of p34^{cdc2}, two consecutive immunoprecipitations were carried out using PC12 lysates (150 μg of total protein), 15 μl of cdc2 sera or normal rabbit sera, and 300 μl of 20% PAS. For immunoblotting, lysates were stored at −80°C before use. Laemmli sample buffer was added to the lysates, and 75 μg of protein was loaded per lane. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon nitrocellulose membrane (Millipore, Bedford, MA) using the Bio-Rad Mini-Protean apparatus, 150 mA, 1 h. Membranes were

blocked, probed, and developed as instructed in the Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL). For cdk2, cdc2, and cyclin A immunoblots, the blocking, probing, and washing was carried out with Tris-buffered saline (TBS) containing 0.05% Tween 20. For the cyclin E blot, TBS with 0.5% Tween 20 was used. Dry milk (5%) was used as the blocking reagent. Primary and secondary antibodies (HRP-conjugated, goat anti-rabbit FAb; Amersham) were diluted in TBS-Tween containing 0.25% dry milk. cdc2, cyclin E, and cyclin A sera were diluted 1:5000; cdk2 antibodies were used at 2 μg/ml.

In Vitro Kinase Assays

Immune complexes were washed with Kinase Buffer (50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol), and kinase assays were carried out as described by Herrmann *et al.* (1991). Immune complexes were incubated in 50-μl reaction containing kinase buffer (1×), 5 μCi γ-^{32}P-ATP (6000 Ci per mmol; New England Nuclear) and 50 mg/ml histone H1 (Boehringer-Mannheim, Mannheim, Germany). The reactions were stopped by the addition of 2× Laemmli sample buffer. Histone H1 was separated from free γ-^{32}P-ATP on 10% polyacrylamide gels. Phosphorylated histone H1 was detected by autoradiography (Kodak X-Omat film, Rochester, NY) and phosphorimaging.

Immunoprecipitation-Electrophoretic Mobility Shift Assay (IP-EMSA)

The DNA sequence of the ^{32}P-labeled probe corresponded to the proximal E2F/DP site found in the adenovirus E2 promoter. The probe was prepared by end-labeling DNA oligonucleotides (5'atttaAGTTTCGCGCCCTTTCTC3' and complementary oligomer), annealling the oligomers, and gel-purifying the double-stranded probe. Cyclin A, cyclin E, p107, and pRB immune complexes were washed with 1× Shift Buffer (20 mM HEPES pH 7.9, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM dithiothreitol). Immunoprecipitated proteins were eluted from the immune complexes using the method of Chellappan *et al.* (1991) with slight modifications: Shift Buffer, 15 μl, containing 0.8% deoxycholate was added to each sample for 10 min on ice and NP40 to a final concentration of 1.2% (1.8 μl) was added, and the samples were kept on ice for an additional 10 min. Ten microliters of the eluted proteins were then combined with 10 μl of the gel shift reaction mix (2× Shift Buffer, 1 μg sheared/sonicated salmon sperm DNA, 30 μg nuclease-free BSA, 2.5% Ficoll, 1.0 μl probe, and when indicated 80 ng competitor oligonucleotide) and incubated on ice for 20 min. Each reaction contained 1.0 μl probe (0.2 ng, 1 × 10^8 cpm/mg). For the electrophoresis reference markers, 10 μg of lysate from PC12-E1A cells (Boulukos and Ziff, 1993) was mixed with 10 μl of the gel shift reaction mix, and the mixture was incubated on ice for 20 min. Ficoll sample buffer (2 μl; 12.5% Ficoll and bromphenol blue) was added to each sample immediately before loading on a TBE-acrylamide gel (0.5% TBE, 4.0% acrylamide). The gels were run at 4°C. The protein-DNA complexes were detected by autoradiography (Kodak X-Omat film) and phosphorimaging.

Peptide Comparison by Partial Proteolysis

Immunoprecipitated proteins were excised from a dry polyacrylamide gel and subjected to proteolysis with V8 protease as described in Harlow and Lane (1988).

Quantitation of Radioactivity

Phosphorimager screens were exposed to dry, radioactive, polyacrylamide gels for 4–24 h. A PhosphorImager (Molecular Dynamics) was used to visualize and quantitate the radioactivity. ImageQuant software was used to quantitate the radioactivty within small defined regions of the gel. For both kinase assays and E2F/DP gel shift assays, radioactivity associated with normal rabbit serum samples was subtracted as background.

Analysis of DNA Content

The procedure of Ignatius *et al.* (1985) with slight modifications was used. This method, in which the DNA content of nuclei rather than whole cells is analyzed, was used to overcome the problem of cell aggregation. Cells were lysed with NP40/PI Solution (0.1% NP40, 50 μg/ml propidium iodide, 100 μg/ml DNase-free RNAseA, 5 mM NaCl, 10 mM trisodium citrate). To avoid aggregation of nuclei, concentrations of ≤10^6 nuclei/ml were maintained. Nuclei were removed from the plates by forceful pipetting. Both the empty plates and nuclei suspensions were examined microscopically to verify that >95% of the nuclei had been removed from the plates and that the nuclei were not aggregating. Within 2 h of cell lysis, the DNA content of individual nuclei was determined by flow cytometry (Becton Dickinson FACScan). The MODFIT program (Modfit Verity Software House, Topsham, ME) was used to quantitate percentages of G0/G1 phase, S phase, and G2/M phase events. Because the nuclear envelope of eukaryotic cells breaks down during M phase (Murray and Hunt, 1993), we have assumed that the G2/M readings reflected the percentage of G2 nuclei and that M phase cells were lost from the population.

RESULTS

NGF Treatment Leads to a Decrease in p33^{cdk2} and p34^{cdc2} Kinase Activity

To analyze the p33^{cdk2} and p34^{cdc2} kinases in PC12 cells, immunological reagents were required that would effectively immunoprecipitate undenatured p33^{cdk2} and p34^{cdc2} from cell extracts and would recognize the denatured proteins on immunoblots. Immunoprecipitations with cdc2 and cdk2 antisera are shown in Figure 1. A polyclonal cdc2 antisera raised in our laboratory and a commercial cdk2 polyclonal antibody each specifically recognized a protein of the appropriate size from

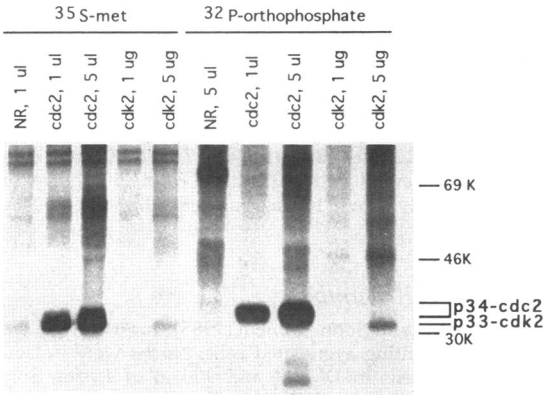

Figure 1. cdc2 and cdk2 antibodies immunoprecipitate proteins from PC12 cells with the characteristics of p34^{cdc2} and p33^{cdk2}. PC12 cells were labeled with ^{35}S-methionine (lanes 1–6) or ^{32}P-orthophosphate (lanes 7–12). The cells were lysed, and immunoprecipitations were carried out with normal rabbit sera (NR) or polyclonal antibodies raised against peptides corresponding to the carboxy terminus of p34^{cdc2} (cdc2) or p33^{cdk2} (cdk2). The immunoprecipitated proteins were resolved on 10% polyacrylamide gels, and the proteins were detected by autoradiography.

^{35}S-methionine labeled and ^{32}P-orthophosphate labeled PC12 cells. Immunoblots of $p33^{cdk2}$ and $p34^{cdc2}$ using the same antisera are shown in Figure 5.

PC12 cell differentiation is characterized by the extension of processes, "neurites" (Greene and Tischler, 1976). Others have shown that the effects of NGF on neurite outgrowth and various aspects of cellular metabolism peak at ~8 d of NGF treatment (Gunning *et al.*, 1981; Burstein and Greene, 1982; Ignatius *et al.*, 1985). For this reason, we carried out each experiment over an 8-d period. PC12 cells were cultured in the presence or absence of NGF. The outgrowth of neurites was observed only in the NGF treated cultures. To determine the effect of NGF on a component of the cell cycle machinery, on day 8 both the untreated and NGF treated cells were lysed. $p33^{cdk2}$ or $p34^{cdc2}$, along with associated cyclins, were immunoprecipitated from the cell extracts. The kinase activity of the immunoprecipitated $p33^{cdk2}$ or $p34^{cdc2}$ was determined in vitro by measuring the level of ^{32}P transferred from γ-^{32}P-ATP to histone H1. Figure 2 shows that the level of both $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity decreased after exposure to NGF. This experiment has been reproduced three times. In each experiment a detectable decrease in the activity of each kinase was observed after 4 d of NGF treatment. After 8 d, a >10-fold decrease in $p33^{cdk2}$ kinase activity and a 2.5-to 3.5-fold decrease in $p34^{cdc2}$ kinase activity were observed. We conclude that the exposure of PC12 cells to NGF leads to the down-regulation of both the $p33^{cdk2}$ and $p34^{cdc2}$ kinases, with the $p33^{cdk2}$ kinase activity decreasing most dramatically.

To determine if the timing of the down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity was dependent on the level of $p140^{trk}$ NGF receptors and the strength of the NGF signal, we analyzed a cell line that overexpresses $p140^{trk}$. A cell line similar to PC12 cells ("PC cells") had been stably transfected with the gene encoding the *trk* NGF receptor. A clonal cell line, 6–24, that overexpresses $p140^{trk}$ had been found to differentiate with accelerated kinetics relative to the parental cells (Hempstead *et al.*, 1992). This suggested that the level of $p140^{trk}$ in the parental cells was a rate-limiting step in differentiation and that an increased number of $p140^{trk}$ receptors was responsible for the accelerated differentiation of the 6–24 cells. We tested whether the 6–24 cells would respond to NGF by down-regulating $p33^{cdk2}$ and $p34^{cdc2}$ kinases with accelerated kinetics. The activity of both kinases was down-regulated within 18 h in the 6–24 cells (Figure 3). This suggested that the timing of the down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity was dependent on the level of $p140^{trk}$ receptors and the strength of the NGF signal.

NGF Regulates Multiple Cyclin-cdk Complexes

$p33^{cdk2}$ and $p34^{cdc2}$ each associate with multiple cyclins during specific phases of the cell cycle to form active

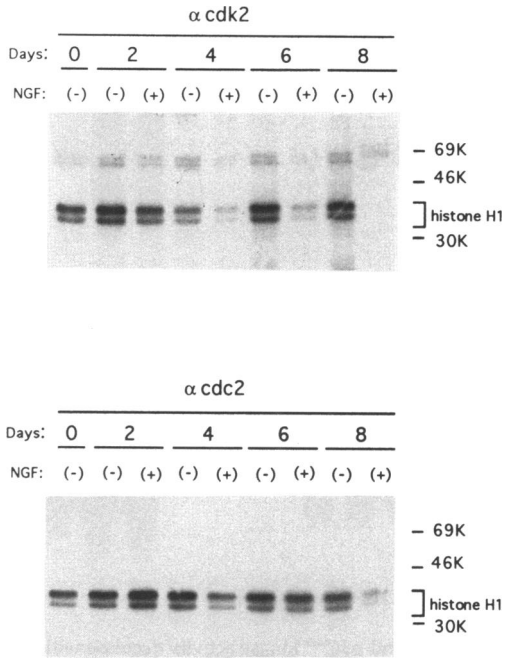

Figure 2. $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity decrease after NGF treatment. PC12 cells were plated every second day for 8 d. One of the cultures from each plating was treated with NGF (50 ng/ml), whereas the other was not. All of the cells were harvested on the same day. Immunoprecipitations with cdk2 (top) and cdc2 (bottom) are shown. In parallel an immunoprecipitation with normal rabbit sera was carried out and used for quantitation (see below). After immunoprecipitation, the immune complexes were incubated with γ-^{32}P-ATP and histone H1 in in vitro kinase assays. The histone H1 was separated from unincorporated γ-^{32}P-ATP by electrophoresis on a 10% acrylamide gel. Histone H1 phosphorylation was detected by autoradiography (top, 60-min exposure; bottom, 10-min exposure) and quantitated by phosphorimaging. The levels of radioactivity in the $p33^{cdk2}$ and $p34^{cdc2}$ samples were 50–500 times higher than in the normal rabbit serum control. After 8 d of NGF treatment, the level of kinase activity immunoprecipitated with cdk2 serum decreased >10 fold, whereas the activity immunoprecipitated with cdc2 serum decreased 2.5- to 3.5-fold.

kinases. $p33^{cdk2}$ associates with A-, D-, and E-type cyclins (Koff *et al.*, 1991; Tsai *et al.*, 1991, 1993; Dulic *et al.*, 1992; Elledge *et al.*, 1992; Rosenblatt *et al.*, 1992; Xiong *et al.*, 1992). Both the cyclinA- and cyclinE-$p33^{cdk2}$ kinases efficiently phosphorylate histone H1 in vitro under our assay conditions. $p33^{cdk2}$ also associates with D-type cyclins (Xiong *et al.*, 1992), but D-type cyclins immunoprecipitated from PC12 cells do not phosphorylate histone H1 above background levels under our assay conditions. This is likely due to the sensitivity of D-type cyclin complexes to the detergent used in the lysis buffer (Matsushime *et al.*, 1992). The regulation of D-type cyclins in PC12 cells will be described elsewhere (Yan and Ziff, unpublished data).

As seen in Figures 2 (PC12 cells) and 3 (6–24 cells), an overall decrease in $p33^{cdk2}$ kinase activity was observed after NGF treatment (Figure 4, lanes 16–20). To

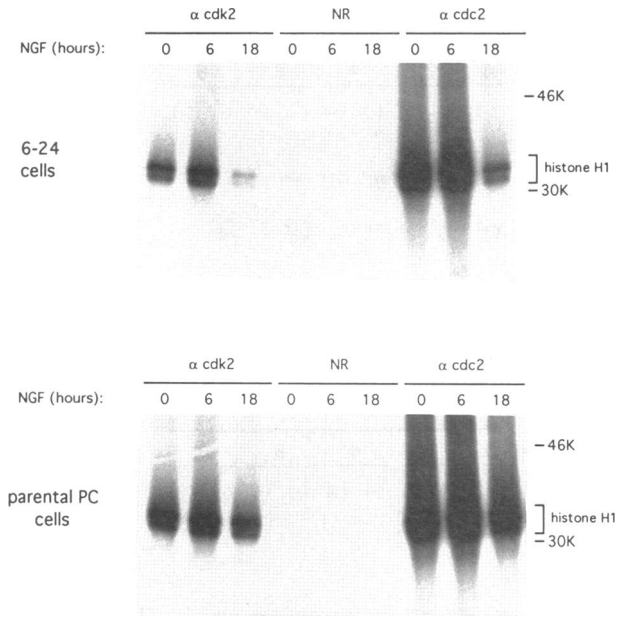

Figure 3. $p33^{cdk2}$ and $p34^{cdc2}$ kinase activity decrease with accelerated kinetics in cells overexpressing $p140^{trk}$. A clonal cell line (6–24; top) that overexpresses the $p140^{trk}$ NGF receptor and differentiates with accelerated kinetics in response to NGF (Hempstead *et al.*, 1992) was treated with NGF for 0, 6, and 18 h. The parental cells were treated in an identical manner (bottom). The cells were lysed, and immunoprecipitations with cdk2 and cdc2 antibodies were carried out. Normal rabbit serum (NR) was used as a negative control. The immune complexes were incubated with γ-^{32}P-ATP and histone H1 in in vitro kinase assays. The histone H1 was separated from unincorporated γ-^{32}P-ATP on a 10% acrylamide gel and detected by autoradiography (5-h exposure).

determine specifically if cyclinE-$p33^{cdk2}$ or cyclinA-$p33^{cdk2}$, or both, were down-regulated during NGF induced differentiation, these cyclins along with associated cdk proteins were immunoprecipitated and assayed for kinase activity. Using polyclonal sera raised against cyclin E or cyclin A, we were able to immunoprecipitate active kinases capable of phosphorylating histone H1. When cyclinE was immunoprecipitated, less cyclinE-associated kinase activity was detected in NGF-treated extracts than in untreated extracts (Figure 4A, lanes 6–10). Cyclin E associates with $p33^{cdk2}$ (Koff *et al.*, 1991; Dulic *et al.*, 1992); thus, the decrease in cyclinE-associated kinase activity most likely reflects a decrease in cyclinE-$p33^{cdk2}$ activity. We conclude that the exposure of PC12 cells to NGF leads to a down-regulation of cyclinE-$p33^{cdk2}$ kinase activity.

The kinase activity immunoprecipitated with cyclin A antisera was also lower in extracts from NGF-treated cells (Figure 4A, lanes 11–15). Because cyclin A associates with two cdk catalytic subunits, both $p33^{cdk2}$ and $p34^{cdc2}$, the kinase activity present in cyclin A immunoprecipitations is expected to be the sum of cyclinA-$p33^{cdk2}$ and cyclinA-$p34^{cdc2}$ activity. To examine the regulation of these two kinases by NGF, the following "preclearing" experiment was carried out. Extracts from NGF treated (5 d) and untreated cells were precleared of cyclinA-$p34^{cdc2}$ kinase using cdc2 antisera and then assayed for cyclinA-$p33^{cdk2}$ kinase activity using cyclin A antisera (Figure 4B). Two consecutive immunoprecipitations with the cdc2 antisera removed >95% of the $p34^{cdc2}$ kinase activity (Figure 4B; compare lanes 1 and 4 for NGF^- extracts; compare lanes 7 and 10 for NGF^+extracts). After the $p34^{cdc2}$ kinase activity was precleared from the extracts, ~60% and 30% of the cyclin A kinase activity remained in the untreated and treated extracts, respectively (Figure 4, B and C; compare lanes 3 and 6 with 9 and 12). Because this cyclinA-associated activity was not due to cyclinA-$p34^{cdc2}$, it is expected to reflect the activity of cyclinA-$p33^{cdk2}$. By subtracting the activity of cyclinA-$p33^{cdk2}$ from the total cyclinA-associated activity, the level of cyclinA-$p34^{cdc2}$ activity was determined. The relative contributions of cyclinA-$p33^{cdk2}$ and cyclinA-$p34^{cdc2}$ to total cyclin A-associated kinase activity was estimated to be 60:40 in untreated extracts and 30:70 in the NGF treated extracts. Both kinases were down-regulated by NGF: cyclinA-$p33^{cdk2}$ activity decreased 80–90%, whereas cyclinA-$p34^{cdc2}$ decreased only 30–40%.

In the same experiment, we measured the kinase activity immunoprecipitated with cyclin B1 antisera. B1-type cyclin associates with one catalytic subunit, namely $p34^{cdc2}$ (Pines and Hunter, 1989). After NGF treatment the cyclinB1-$p34^{cdc2}$ activity decreased 20% (Figure 4, B and C; compare lanes 13 and 15).

From these experiments, we conclude that NGF treatment of asynchronously growing PC12 cells leads to a decrease in the kinase activity of multiple cyclin-dependent kinases. The most dramatic decrease in kinase activity was observed for the $p33^{cdk2}$ kinases, namely cyclinE-$p33^{cdk2}$ and cyclinA-$p33^{cdk2}$. These kinases were down-regulated to a larger extent than the $p34^{cdc2}$ kinases.

NGF Treatment Leads to a Decrease in the Steady-State Levels of $p33^{cdk2}$ and $p34^{cdc2}$

To determine if the observed down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$kinase activities correlated with a decrease in the steady-state level of the cyclin and/or cdk proteins, we analyzed these proteins by immunoblotting (Figure 5). The same extracts used for analyzing kinase activity (Figure 4A) were analyzed for protein levels (Figure 5). The levels of cyclin A and cyclin E did not change appreciably after 6–8 d of NGF treatment. However, the levels of both $p33^{cdk2}$ and $p34^{cdc2}$ decreased after NGF treatment. It is likely that these decreases in the steady-state levels of $p33^{cdk2}$ and $p34^{cdc2}$ contributed to the decreases in their kinase activity. By densitometric scanning of the immunoblot autoradiograms, we determined that the relative decreases in

p33^{cdk2} and p34^{cdc2} after NGF treatment were very similar. Thus, the greater decrease in p33^{cdk2} kinase activity relative to p34^{cdc2} is not explained by the relative decreases in protein levels (see DISCUSSION).

NGF Treatment Results in a Decrease in p107-associated Kinase Activity

p33^{cdk2} has been identified in complexes with the retinoblastoma protein (pRb) related protein p107, cyclins, and transcription factors of the E2F/DP family (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992). We have investigated the effect of NGF on the regulation of p107 protein levels, its association with a histone H1 kinase activity, and its association with E2F/DP. As determined by ^{35}S-methionine labeling of PC12 cells and immunoprecipitation with p107 antibodies, the level of p107 remained relatively constant even after 7 d of NGF treatment (Figure 6A). In contrast, a decrease in the level of p107-associated histone H1 kinase activity was detected within 3.5 d of NGF treatment (Figure 6B). This decrease paralleled a decrease in p33^{cdk2} kinase activity, as would be expected if the p107-associated kinase were p33^{cdk2} (Figures 2 and 6B). In Figure 6C, cell extracts were immunoprecipitated with a p107 or a p33^{cdk2} antibodies and the immune complexes were incubated with γ-^{32}P ATP and fractionated by SDS-PAGE. A band corresponding to in vitro kinase labeled p107 is seen in the αp107 immunoprecipitation lane. A band in the αp33^{cdk2} immunoprecipitation lane that comigrated with p107 was excised and in Figure 6D was found by partial proteolyic peptide mapping to be identical to authentic p107. This experiment demonstrated that immunoprecipitation using p33^{cdk2} antibodies co-immunoprecipitated p107. Thus, it is likely that the NGF-sensitive kinase activity that is associated with p107 in PC12 cells is p33^{cdk2}. A decrease in p107-E2F/DP-DNA complexes was also observed after NGF treatment (see below, Figure 7A). Thus, NGF regulates the formation of a complex(es) containing p107, an active kinase, and an active transcription factor. We have not determined if this is due to a decrease in the binding of p107 to p33^{cdk2} and E2F/DP or to an inactivation of the intact complex(es).

NGF Regulates E2F/DP Transcription Factor Complexes

Cyclin A-p33^{cdk2} and cyclinE-p33^{cdk2} kinases form stable complexes with transcription factors of the E2F/DP family and with the tumor-suppressor-like protein p107 (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992). These multicomponent transcription factor complexes have been observed in extracts from a wide range of cell types (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992). We have identified cyclinA-, cyclinE-, and p107-containing E2F/DP complexes using an immunoprecipitation-electrophoretic motility shift assay (IP-EMSA). With this assay, proteins immunoprecipitated with cyclin A, cyclin E, or p107 antisera were eluted from the immune complexes with detergent. The eluted proteins were mixed with ^{32}P-labeled DNA oligomers containing a binding site for E2F/DP transcription factors, and the E2F/DP-DNA complexes were resolved on a nondenaturing gel as in a standard gel shift assay. In each case the eluted proteins displayed the DNA binding specificity of E2F/DP transcription factors, in that they bound to the probe in the presence of mutant (M) but not wild-type (W) DNA competitor (Figure 7A, lanes 8–9, 16–17, and 23–24).

To determine if these complexes were regulated by NGF, extracts from NGF-treated and untreated cells were analyzed for the presence of cyclin A-, cyclinE-, and p107-associated E2F/DP. The same extracts used to measure kinase activity (Figure 4A) and protein levels (Figure 5) were assayed here for E2F/DP (Figure 7A). The E2F/DP DNA-binding activity immunoprecipitated with p107 sera and cyclin A sera decreased after NGF treatment (Figure 7A, lanes 16–22 and 23–29, respectively), whereas the E2F/DP DNA-binding activity immunoprecipitated with cyclin E sera did not change (Figure 7A, lanes 8–14). Thus, the cyclin A- and p107-containing complexes were sensitive to NGF treatment, whereas the cyclinE-containing complex was not.

The retinoblastoma protein (pRb), which shares large regions of amino acid homology with p107, also forms a complex with an E2F/DP DNA binding activity in PC12 cells. The pRB-associated E2F/DP activity was detected in extracts of undifferentiated PC12 cells using the IP-EMSA (Figure 7B, lane 2). Interestingly, the E2F/DP activity that was eluted from pRB ("E2F/DP$_{pRB}$") migrated faster in the nondenaturing gel than the E2F/DP activity eluted from cyclin A, cyclin E, and p107 immune complexes ("E2F/DP$_{p107/cyA/cyE}$"; Figure 7, A and B). This suggested that the E2F/DP species associated with cyclin A, cyclin E, and p107 was distinct from the pRB-associated species. It is possible that the E2F component of the DNA-binding activity is different, because several members of the E2F family of proteins preferentially bind to either pRB or p107 (Dyson *et al.*, 1993; Lees *et al.*, 1993). When the pRB-associated DNA binding activity was analyzed in extracts from untreated and NGF-treated cells by IP-EMSA, the level of pRB-E2F/DP was similar (Figure 7C). Thus, the pRB-E2F/DP complex was insensitive to NGF treatment.

NGF Leads to a Decrease in 2n and 3n Cells Accompanied by an Increase in 4n Cells

We have shown that the expression and activity of p33^{cdk2} and p34^{cdc2} kinases, key components of cell cycle controls, are regulated during PC12 cell differentiation. In addition, we have demonstrated that the cyclin

A- and p107-associated E2F/DP DNA binding activity, which has been observed in S phase and M phase cells (Lees *et al.*, 1992; Shirodkar *et al.*, 1992) and G0/G1 and S phase cells (Devoto *et al.*, 1992; Lees *et al.*, 1992; Cobrinik *et al.*, 1993), respectively, decreases after NGF treatment. We next sought to determine if these biochemical changes would be manifested in altered progression through the phases of the cell cycle. Thus, we

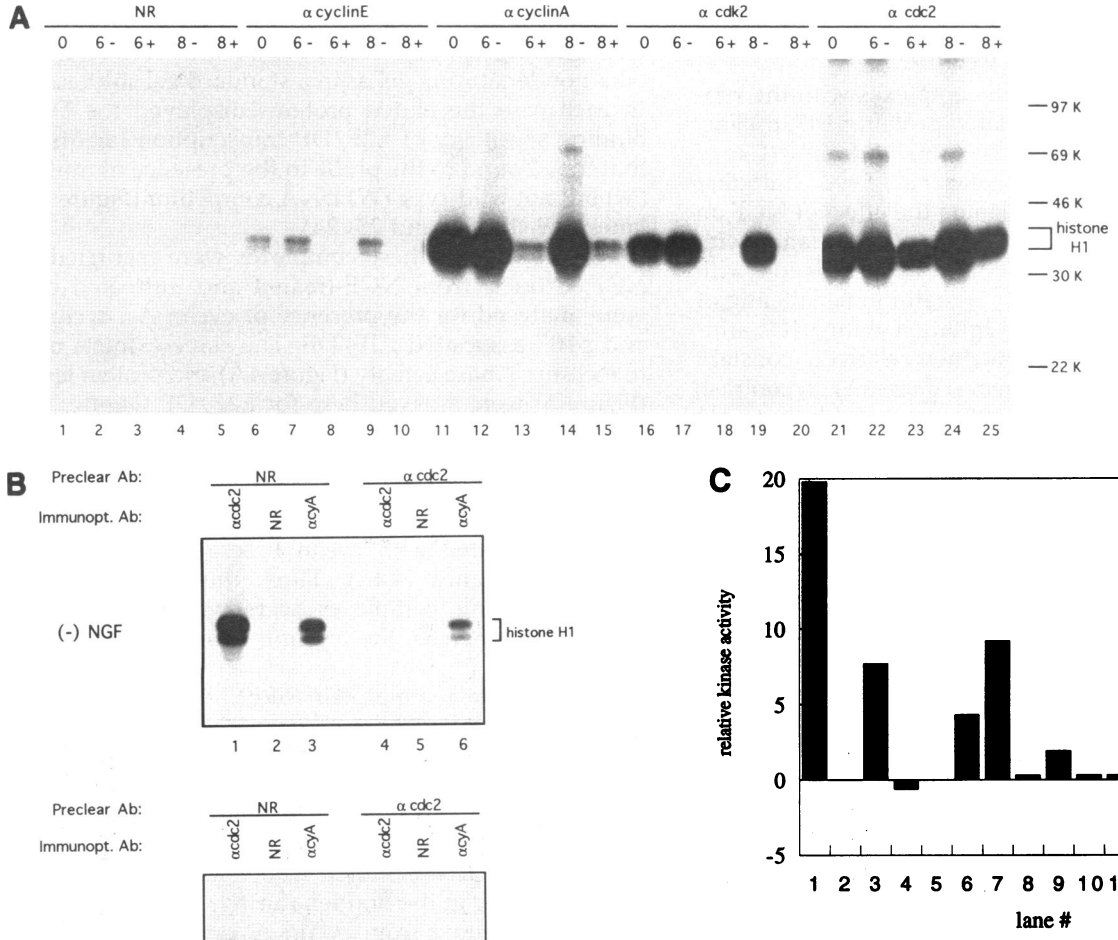

Figure 4. The kinase activities of multiple cyclin-cdk kinases decrease after NGF treatment. (A) PC12 cells were cultured in the absence (−) or presence (+) of NGF for 0, 6, and 8 d. The cells were lysed, and equal amounts of each lysate (100 mg protein/sample) were used in immunoprecipitations with normal rabbit serum (NR; lanes 1–5), cyclin E (lanes 6–10), cyclin A (lanes 11–15), cdk2 (lanes 16–20), or cdc2 (lanes 21–25) polyclonal antibodies. In vitro kinase assays were carried out as in Figures 2 and 3. The kinase activity immunoprecipitated with each antisera was detected by autoradiography (2-h exposure) and quantitated by phosphorimaging. The decrease in kinase activity in NGF-treated samples relative to untreated samples (day 8) was ~2- to 7-fold for a cyclin E, 10- to 13-fold for a cyclin A, 20- to 50-fold for a cdk2, and 2- to 3-fold for a cdc2. (B and C) PC12 cells were cultured for 5 d in the absence (lanes 1–6, 13–14) or presence (lanes 7–12, 15–16) of NGF. The cells were lysed. For lanes 1–12, equal amounts of lysate were used in two consecutive immunoprecipitations with normal rabbit sera (NR) or cdc2 sera (a cdc2). The proteinA-Sepharose was discarded, and the lysates, now "precleared" of kinase activity associated with cdc2 sera (lanes 4–6, 10–12) or normal rabbit sera (lanes 1–3, 7–9), were divided for the final immunoprecipitation. An equal amount of the precleared lysates (25 mg protein/sample) was used in immunoprecipitations with cdc2 (lanes 1, 4, 7, 10), normal rabbit (lanes 2, 5, 8, 11), or cyclin A (lanes 3, 6, 9, 12) sera. After the final immunoprecipitation, in vitro kinase assays were carried out as in Figures 2 and 3. For lanes 13–15, equal amounts of lysate (25 mg protein/sample) were used in immunoprecipitations with cyclin B1 sera (lanes 13 and 15) or normal rabbit sera (lanes 14 and 16), and the immune complexes were assayed for kinase activty in vitro. Histone H1 phosphorylation was detected by autoradiography (2-h exposure) and quantitated by phosphorimaging (C).

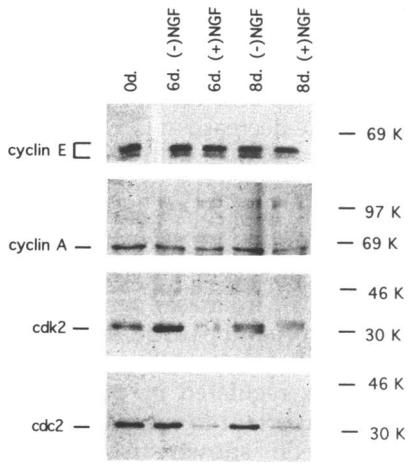

Figure 5. The steady-state levels of p33^{cdk2} and p34^{cdc2} decrease after NGF treatment. PC12 cells were cultured in the absence (−NGF) or presence (+NGF) of nerve growth factor for 0, 6, and 8 d. The cells were lysed, and an equal amount of lysate (75 mg protein) was loaded per lane on a 10% polyacrylamide gel. The proteins were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was probed with cyclin E, cyclin A, cdk2, or cdc2 polyclonal antibodies, as indicated, followed by horseradish peroxidase (HRP)-conjugated secondary antibody. The HRP-immune complexes were detected using an enhanced chemiluminescence kit.

analyzed the proliferation rates and cell cycle distribution of cells. To determine the effect of NGF treatment on proliferation rates, we analyzed the mean population doubling times of the cells in the absence and presence of NGF. For cells cultured under standard conditions (15% serum), the mean population doubling time from day 0–8 was longer in the presence of NGF than in the absence, but neither culture reached a zero growth plateau (Figure 8). Similar results were obtained when the cells were cultured in lower serum concentrations of 5% and 7.5%. This demonstrated that NGF did not completely block the proliferation of the PC12 cells.

To determine if NGF affected the cell cycle phase distribution of the cells, the DNA content of the cells was measured. Nuclei were isolated from cells cultured under standard conditions in the presence or absence of NGF for 0–8 d. Nuclei rather than whole cells were analyzed to overcome the problem of cellular aggregation. The nuclei were stained with propidium iodide and analyzed by flow cytometry. The distribution of nuclei in the 2n peak (G0/G1 phase), the 3n area (S phase), and the 4n peak (G2 phase) of the flow cytometry histograms was quantitated. The percentage of 4n M phase cells was not determined by this method, because the nuclear envelope of multicellular eukaryotes breaks down during M phase (Murray and Hunt, 1993). Flow cytometry histograms of nuclei harvested from cells that had been treated with NGF for 0 and 8 d are shown in Figure 9A. The graphs in Figure 9B show the phase distribution of nuclei harvested after 0, 2, 4, 6, and 8 d of cell culture in the absence and presence of NGF. The experimental protocol, which involved plating the cells on collagen-coated dishes 2 d before the addition of NGF-supplemented media (or for controls, NGF-minus media), resulted in an increase in 2n cells in the population by day 2 of the experiment. However, this increase in 2n cells was observed in both the untreated and NGF-treated cultures and thus was not a specific effect of NGF. When the phase distribution of cells cultured in the absence or presence of NGF for 8 d was compared, we observed a decrease in the percentage of 2n nuclei in the NGF-treated cells (58%) relative to the untreated cells (72%) and a decrease in the percentage of 3n nuclei in the NGF-treated cells (10%) relative to the untreated cells (20%). An accompanying increase in 4n nuclei was observed in the NGF-treated cells (35%) relative to the untreated cells (10%). In summary, NGF treament resulted in a decrease in 2n and 3n nuclei with an accompanying increase in 4n nuclei. These data are very similar to the data of others (Ignatius *et al.*, 1985).

DISCUSSION

Regulation of cdk Kinases During PC12 Differentiation

We have shown that NGF treatment of PC12 cells leads to a reduction in the level as well as the enzymatic activity of p33^{cdk2} and p34^{cdc2} kinases. After NGF treatment, the steady-state protein levels of p33^{cdk2} and p34^{cdc2} decline along with the activity of cyclinA-p34^{cdc2}, cyclinB-p34^{cdc2}, cyclinE-p33^{cdk2}, and cyclinA-p33^{cdk2} kinases. For each of these complexes, the decrease in kinase activity can be attributed at least in part to a decrease in the level of the catalytic subunit. The levels of cyclin A and E were not affected by NGF.

It is interesting that the protein levels of the catalytic subunits, rather than the cyclins, were regulated during PC12 differentiation, because this is the opposite of what has been observed during cell cycling. Proliferating cells that are progressing through the phases of the cell cycle maintain relatively constant levels of p33^{cdk2} and p34^{cdc2} (Draetta and Beach, 1988; Marraccino *et al.*, 1992; Rosenblatt *et al.*, 1992), whereas the levels of cyclin A, B, and E fluctuate dramatically (Dulic *et al.*, 1992). The down-regulation of p33^{cdk2} and p34^{cdc2} protein levels and kinase activity during PC12 differentiation is similar to what has been observed during the growth arrest of fibroblasts and epithelial cells (Draetta *et al.*, 1988; Lee *et al.*, 1988; Tsai *et al.*, 1993). The levels of p33^{cdk2} and p34^{cdc2} protein (nuclear) and kinase activity also decrease during the differentiation of myotubes (Cardoso *et al.*, 1993; Cardoso, personal communication). Thus, a decline in p33^{cdk2} and p34^{cdc2} protein levels is associated with proliferative arrest. Although NGF-treated PC12 cells did not completely arrest proliferation, the doubling times of the cell population did

increase almost twofold. This was likely the result of the down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ kinases.

When fibroblasts and macrophages are deprived of growth factors, the steady-state levels of cdk2 and cdc2 mRNAs decrease (Lee *et al.*, 1988; Matsushime *et al.*, 1992). Our laboratory has shown that cdk2 and cdc2 mRNA levels also decline after NGF treatment of PC12 cells (Yan and Ziff, unpublished data). Thus, the mechanism(s) leading to the decline of $p33^{cdk2}$ and $p34^{cdc2}$ protein levels during PC12 differentiation may be similar to the mechanisms regulating their expression during proliferative arrest induced by growth factor starvation.

It is likely that a regulatory mechanism(s) in addition to decreased expression of the catalytic subunit is impinging on the activity of $p33^{cdk2}$ kinase. Although comparable decreases in the steady-state protein levels of $p33^{cdk2}$ and $p34^{cdc2}$ were observed after NGF treatment, the activity of $p33^{cdk2}$ kinase decreased to a greater extent. Within 5–6 d of exposure to NGF, cyclinA-$p33^{cdk2}$ and cyclinE-$p33^{cdk2}$ activity each decreased 80–90%, whereas cyclinA-$p34^{cdc2}$ and cyclinB1-$p34^{cdc2}$ activity was reduced only 30–40% and 20%, respectively. Thus, we speculate that an inhibitory mechanism specific to $p33^{cdk2}$ is induced by NGF and contributes to the dramatic decrease in kinase activity. It has been demonstrated in several types of cells that $p33^{cdk2}$ kinases can be negatively regulated by phosphorylation (Gu *et al.*, 1992) and the binding of inhibitory molecules (Gu *et al.*, 1993; Gyuris *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Polyak *et al.*, 1994). It will be of interest to determine if these mechanisms contribute to a reduction in kinase activity in PC12 cells.

Surprisingly, the level of cyclin A did not decrease significantly during PC12 differentiation. During myogenic differentiation and the growth arrest of fibroblasts, cyclin A protein levels decline (Cardoso *et al.*, 1993; Tsai *et al.*, 1993). Although the levels of cyclin A did not change significantly during PC12 cell differentiation, the association of cyclin A with an E2F/DP DNA-binding activity did. Thus, the expression and activity of cyclin A is regulated differently during PC12 cell differentiation than during proliferative arrest.

Regulation of Transcription Factor Complexes During PC12 Cell Differentiation

We have shown that NGF treatment leads to a decrease in cyclinA- and p107-associated E2F/DP DNA binding activity. When measuring cyclinA-associated E2F/DP DNA binding activity in the IP-EMSA assay, a decrease in activity could theoretically result from a decrease in the level of cyclinA or E2F/DP protein, a decrease in their association with each other (which in some complexes may require additional proteins), or a decrease in the association of E2F/DP with the DNA. Similarly, a decrease in p107-associated E2F/DP DNA binding activity could result from a decrease in the level of p107 or E2F/DP, a decrease in their association, or a decrease in the association of E2F/DP with DNA.

We have demonstrated that the levels of cyclin A and p107 proteins in PC12 cells remain constant after NGF treatment. Thus, the decrease in E2F/DP DNA binding activity immunoprecipitated with cyclin A antibodies is not due to a decrease in cyclin A protein levels. Similarly, the decrease in E2F/DP DNA binding activity immunoprecipitated with p107 antibodies is not due to a decrease in p107 protein levels. This suggests other levels of regulation. Because a kinase (namely $p33^{cdk2}$) is associated with cyclin A and p107 in the undifferentiated cells, it is possible that the stability of the transcription factor complexes is regulated by phosphorylation. In this respect it is important to note that both p107 and E2F/DP have been shown to be substrates of cyclinA-$p33^{cdk2}$ (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Peeper *et al.*, 1993). The regulation of cyclinA- and p107-E2F/DP complexes by phosphorylation is under investigation.

Cyclin A has been identified in complexes with p107, $p33^{cdk2}$, and E2F/DP (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992). Cyclin A has also been found in association with p130 (a p107-related protein), $p33^{cdk2}$, and E2F/DP (Cobrinik, *et al.* 1993; Hannon *et al.*, 1993; Li *et al.*, 1993), although the association of all of these components in one complex has not been demonstrated. Cyclin A has also been shown to interact with E2F/DP in a p107- and p130-independent manner (Krek *et al.*, 1994). Thus, cyclin A may exist in at least three different E2F/DP complexes, one involving p107, one involving p130 and one lacking both of these "pocket proteins."

Others have shown that high-affinity binding of p107 to cyclinA requires $p33^{cdk2}$ (Peeper *et al.*, 1993). Therefore, one would predict that a decrease in $p33^{cdk2}$ protein levels, as we have observed in NGF-treated PC12 cells, would result in a decrease in p107-dependent, cyclinA-E2F/DP association. We have observed that NGF leads to a decrease in both $p33^{cdk2}$ protein levels and cyclinA-associated E2F/DP activity; thus, we speculate that the decrease in $p33^{cdk2}$ protein is contributing to the decrease in cyclinA-associated E2F/DP activity by destabilizing cyclinA-pocket protein-E2F/DP-DNA complexes. It is also possible that NGF destabilizes other cyclinA-E2F/DP complexes, such as those lacking pocket proteins.

It is of particular interest that the cyclinA- and cyclinE-E2F/DP complexes are regulated differently after NGF treatment. Cyclin E, like cyclin A, has been shown to interact with E2F/DP in gel shift complexes containing $p33^{cdk2}$ and a pocket protein (Cao *et al.*, 1992; Devoto *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). However, the requirement for $p33^{cdk2}$ in stabilizing cyclinE-pocket protein complexes has not been demonstrated to date, whereas it has for cyclinA-pocket protein complexes (Peeper *et al.*, 1993).

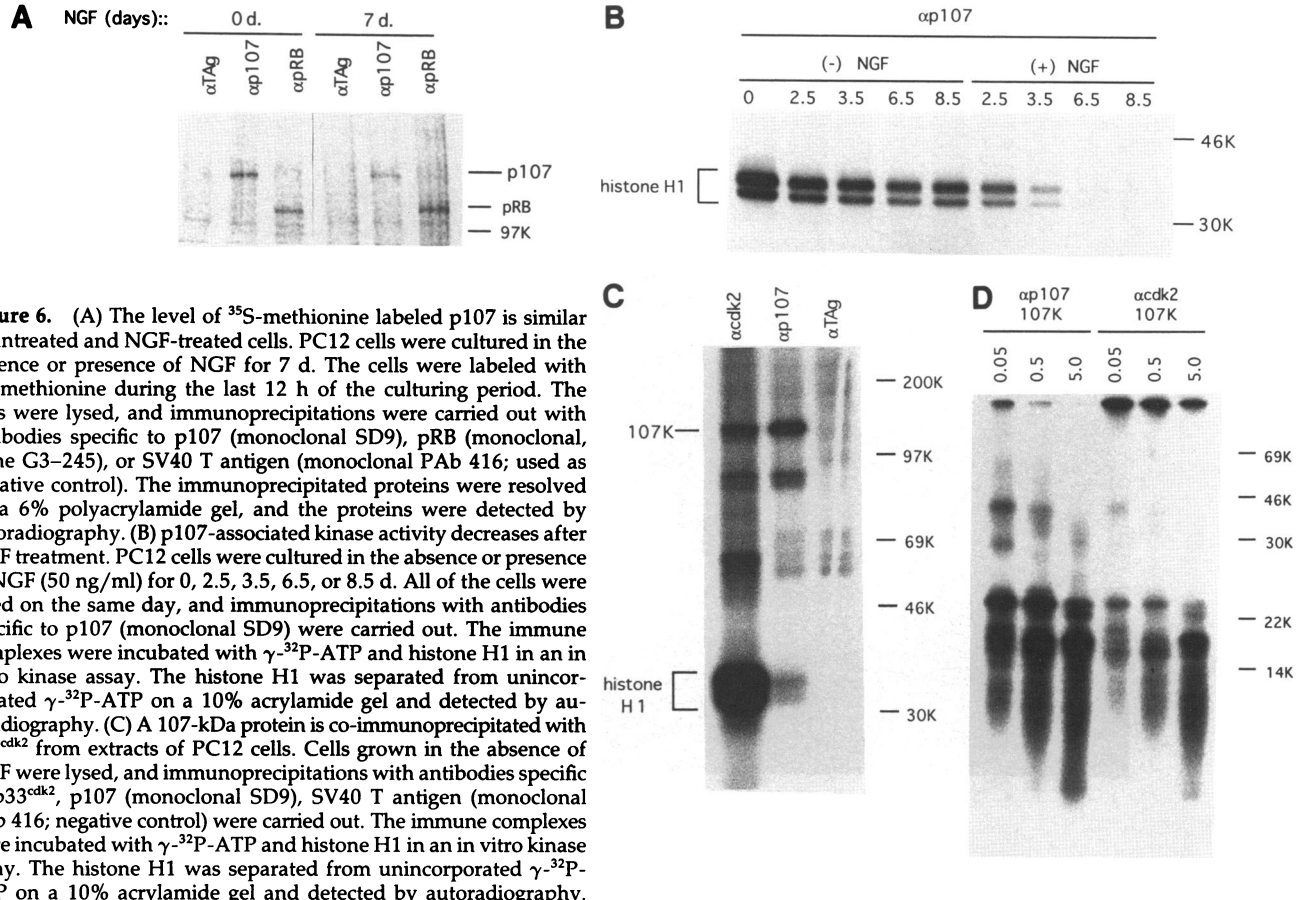

Figure 6. (A) The level of ^{35}S-methionine labeled p107 is similar in untreated and NGF-treated cells. PC12 cells were cultured in the absence or presence of NGF for 7 d. The cells were labeled with ^{35}S-methionine during the last 12 h of the culturing period. The cells were lysed, and immunoprecipitations were carried out with antibodies specific to p107 (monoclonal SD9), pRB (monoclonal, clone G3–245), or SV40 T antigen (monoclonal PAb 416; used as negative control). The immunoprecipitated proteins were resolved on a 6% polyacrylamide gel, and the proteins were detected by autoradiography. (B) p107-associated kinase activity decreases after NGF treatment. PC12 cells were cultured in the absence or presence of NGF (50 ng/ml) for 0, 2.5, 3.5, 6.5, or 8.5 d. All of the cells were lysed on the same day, and immunoprecipitations with antibodies specific to p107 (monoclonal SD9) were carried out. The immune complexes were incubated with γ-^{32}P-ATP and histone H1 in an in vitro kinase assay. The histone H1 was separated from unincorporated γ-^{32}P-ATP on a 10% acrylamide gel and detected by autoradiography. (C) A 107-kDa protein is co-immunoprecipitated with p33^{cdk2} from extracts of PC12 cells. Cells grown in the absence of NGF were lysed, and immunoprecipitations with antibodies specific to p33^{cdk2}, p107 (monoclonal SD9), SV40 T antigen (monoclonal PAb 416; negative control) were carried out. The immune complexes were incubated with γ-^{32}P-ATP and histone H1 in an in vitro kinase assay. The histone H1 was separated from unincorporated γ-^{32}P-ATP on a 10% acrylamide gel and detected by autoradiography. (D) The 107-kDa protein associated with p33^{cdk2} is p107. The 107-kDa proteins immunoprecipitated with p107 antibodies or p33^{cdk2} antibodies (C) were excised from the gel and subjected to proteolysis with *S. aureus* V8 protease. The proteins were digested with 0.05, 0.5, 5.0 mg V8 protease per lane.

This allows for the possibility that cyclinE-pocket protein-E2F/DP complexes are stable in the absence of p33^{cdk2} and provides a possible explanation for the differential regulation of cyclinA- and cyclinE-E2F/DP complexes after NGF treatment.

Cyclin E, unlike cyclin A, has not been shown to bind E2F/DP in a pocket protein-independent manner. This suggests that the cyclinE-E2F/DP complexes observed in PC12, those stable in NGF-treated cells, contain a pocket protein.

Preferential Accumulation of PC12 Cells with a 4n DNA Content

As is evident from the growth curves and flow cytometry analysis (Figures 8 and 9), NGF caused neither a rapid nor a complete arrest of PC12 cell cycling. However, NGF treatment did lead to a decrease in the percentage of 2n and 3n cells and an increase in the percentage of 4n cells. These data are in agreement with the results of Ignatius *et al.* (1985). Rudkin *et al.* (1989) have reported an increase in 2n cells from 55% to 70% of the cell population within 1 d of NGF treatment. We have observed a similar increase in 2n cells within 2 d of NGF treatment, but under our culturing conditions, this increase was observed even in the absence of NGF (Figure 9B) and thus was not an NGF-specific effect. An NGF-induced accumulation of 2n cells was not observed.

Based on the biochemical data that demonstrated that NGF caused a decrease in the expression and activity of p33^{cdk2} and p34^{cdc2}, one would predict a decrease in S phase cells and an accompanying accumulation of cells at the G1/S and G2/M transition points. This prediction is based on evidence that p33^{cdk2} and p34^{cdc2} are required in other cell types for passage through the G1/S and G2/M transition points, respectively (Riabowal *et al.*, 1989; Pagano *et al.*, 1993; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993). Although the predicted decrease in 3n (S phase) and the predicted increase in 4n (G2 phase) cells were observed, an accumulation of 2n cells indicative of G0/G1 cells was not observed. It is somewhat paradoxical that although p33^{cdk2} (a cdk shown to function primarily at the

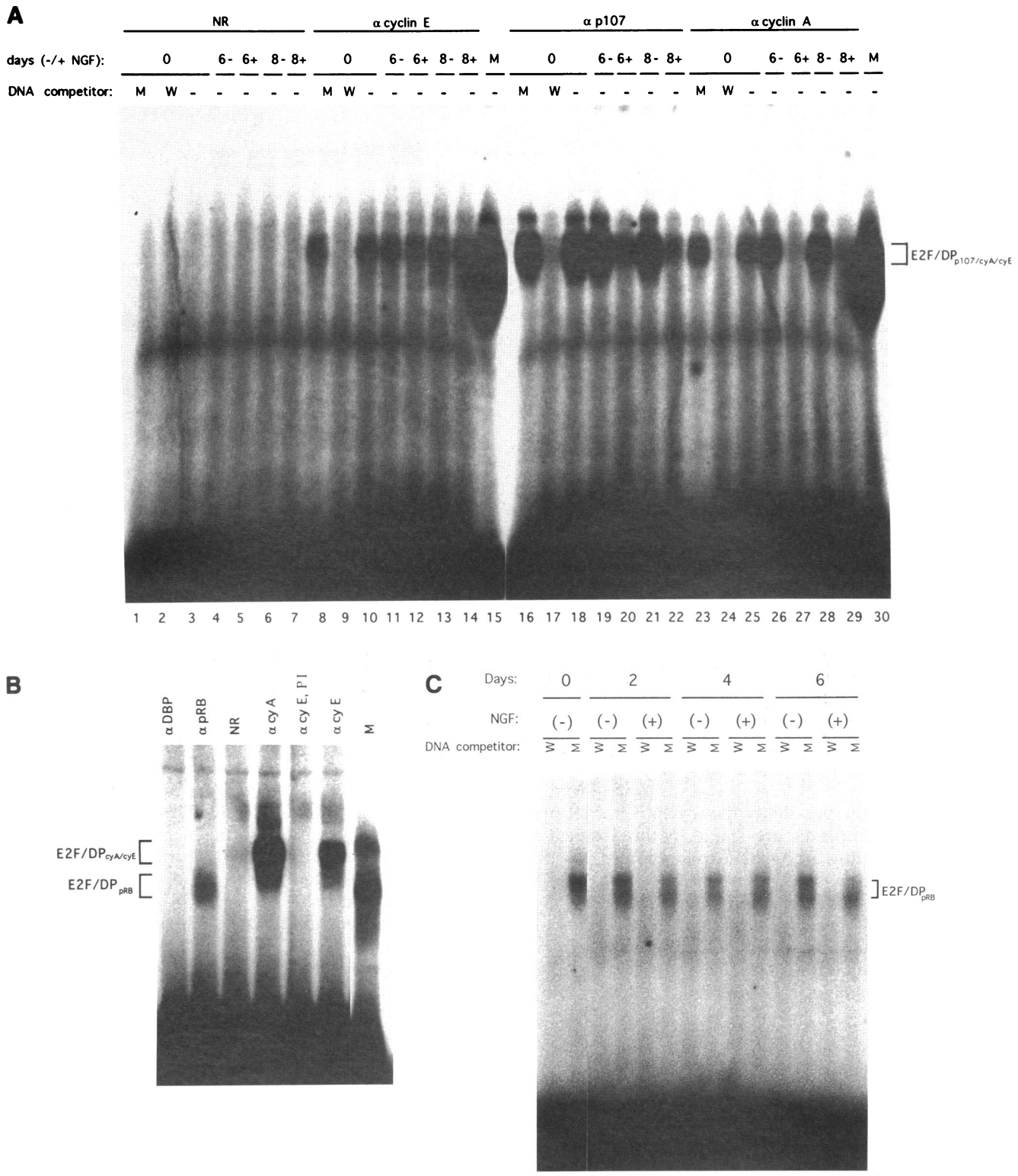

Figure 7. CyclinA- and p107-E2F/DP, but not cyclinE- or pRB-E2F/DP, transcription factor complexes are sensitive to NGF. (A) PC12 cells were cultured for 0, 6, or 8 d in the absence or presence of NGF. The cells were lysed, and equal amounts of lysate (125 mg protein/sample) were used in immunoprecipitations with normal rabbit (NR; lanes 1–7), cyclin E (lanes 8–14), p107 (lanes 16–22), or cyclin A (lanes 23–29) sera. Immunoprecipitated proteins were eluted from the immune complex with deoxycholate. The eluted proteins were mixed with a ^{32}P-labeled DNA oligomer containing an E2F/DP binding site and incubated on ice. Protein-DNA complexes were separated from unbound DNA

G1/S transition) was down-regulated to a larger extent than p34^{cdc2} (a cdk shown to function primarily at the G2/M transition), a greater increase in the percentage of 4n, G2 phase cells rather than 2n, G0/G1 phase was observed. The reason for this preferential accumulation of 4n cells is not clear. We have considered the possibility that another G1-phase cyclin-dependent kinase was substituting for p33^{cdk2}, allowing cells to pass through the G1/S transition. However, studies in other cell types with dominant negative p33^{cdk2} mutants and antibody microinjection experiments have indicated that blocking p33^{cdk2} activity is sufficient to induce a G0/G1 phase arrest (Pagano *et al.*, 1993; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993). Others have noted a drift toward polyploidy in PC12 cells after several weeks of NGF treatment (Ignatius *et al.*, 1985). Therefore, we are considering the possibility that a fraction of the cells that accumulate with a 4n DNA content after NGF treatment are tetraploid cells arrested in G0/G1 phase and thus not detected as 2n cells. At this time, it still remains to be determined why the NGF-treated PC12 cells do not accumulate with a 2n DNA content.

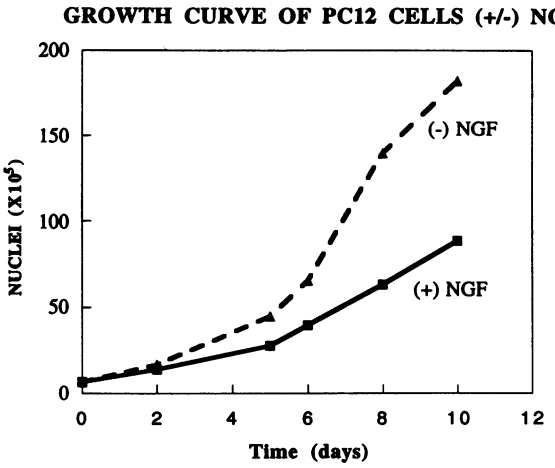

Figure 8. Proliferation rates of PC12 cells in the presence and absence of NGF. PC12 cells were plated (2×10^5 cells/60-mm plate) and cultured in the presence (——) or absence (– – –) of NGF. After 0, 2, 5, 6, 8, and 10 d of NGF treatment, the cells were lysed and nuclei were counted. Nuclei, rather than cells, were analyzed, because cell aggregation interfered with counting cells. The mean population doubling time of the cells was longer in the presence of NGF (74 h) than in the absence (46 h).

Future Questions and/or Studies

In this study we have demonstrated that several key components of the cyclin-cdk signaling loop are regulated by NGF. We have not, however, determined precisely where the NGF signaling pathway intersects with the cyclin-cdk signaling loop at the molecular level. To determine this, it will be necessary to determine the *temporal order* of the biochemical events observed after NGF treatment. Feedback regulatory loops that allow "early" (G1 and S phase) cyclin-cdk complexes to regulate "late" (G2 and M phase) cyclin-cdks, and vice versa, have been demonstrated in yeast (Amon *et al.*, 1993) and are likely to exist in multicellular eukaryotes. In addition, the regulation of an S phase cdk kinase by G1 phase cdk kinases has been demonstrated in mammalian cells (Ewen *et al.*, 1993). Thus, although multiple cyclin-cdk kinases are regulated during PC12 cell differentiation, some may be linked more directly to the NGF signaling pathway than others. Experiments with a synchronized population of cells will be required to determine the temporal order of the biochemical events observed after NGF treatment.

Relevance to the Development of NGF-dependent Neurons In Vivo

PC12 cells were derived from a pheochromocytoma, a tumor originating from adrenal medullary chromaffin cells. Like sympathetic neurons, chromaffin cells are of neural crest origin. After exposure to NGF, PC12 cells develop a number of properties characterisitc of sympathetic neurons, including electrical excitability and the expression of neuronal-specific genes. In this study we have demonstrated that the expression and activity of

probe by electrophoresis through a nondenaturing polyacrylamide gel. As electrophoresis reference markers (M; lanes 15 and 30), protein-DNA complexes that resulted from mixing 10 mg of lysate from gE1a-PC12 cells with ^{32}P-labeled probe, were loaded on the gel. On shorter exposures, two complexes were apparent in these lanes (as seen in B). The more slowly migrating complex co-migrates with the E2F/DP species immunoprecipitated with p107, cyclin A, and cyclin E sera ("E2F/DP$_{p107/cyA/cyE}$"). (B) The IP-EMSA assay was carried out as described in A using extracts from undifferentiated cells. Antibodies specific to the retinoblastoma protein (pRB), cyclin A, and cyclin E were used. Normal rabbit (NR) sera, preimmune sera (PI), and antibodies raised against a viral protein (αDBP) were used as negative controls. The electrophoresis marker (M) was generated by mixing 10 mg of lysate from gE1a-PC12 cells with ^{32}P-labeled probe in a standard gel shift reaction. This yielded two complexes that represent "free E2F/DP", that is, complexes from which the p107 and pRB have been dislodged by the adenovirus E1A protein (Bagchi *et al.*, 1990). One complex co-migrates with the E2F/DP species immunoprecipitated with cyclin A and cyclin E sera ("E2F/DP$_{cyA/cyE}$"); the other co-migrates with the E2F/DP species immunoprecipitated with sera specific for the retinoblastoma protein, pRB ("E2F/DP$_{pRB}$"). (C) PC12 cells were cultured for 0, 2, 4, or 6 d in the absence or presence of NGF. The cells were lysed, and equal amounts of lysate (125 mg protein/sample) were used in immunoprecipitations with monoclonal antibodies specific to the retinoblastoma protein. Immunoprecipitated proteins were eluted from the immune complex with deoxycholate. The eluted proteins were mixed with a ^{32}P-labeled DNA oligomer containing an E2F/DP binding site and incubated on ice. Protein-DNA complexes were separated from unbound DNA probe by electrophoresis through a nondenaturing polyacrylamide gel.

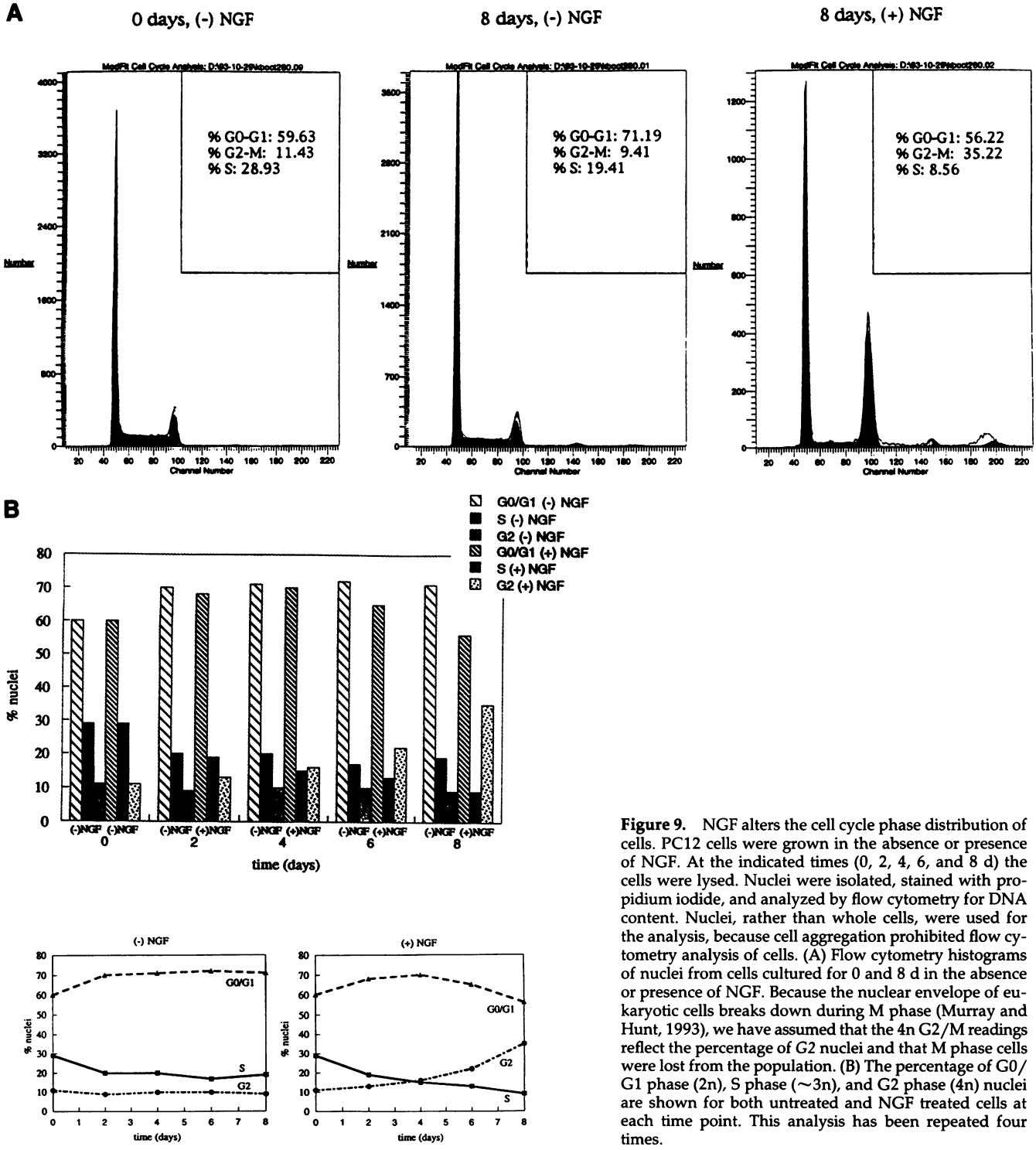

Figure 9. NGF alters the cell cycle phase distribution of cells. PC12 cells were grown in the absence or presence of NGF. At the indicated times (0, 2, 4, 6, and 8 d) the cells were lysed. Nuclei were isolated, stained with propidium iodide, and analyzed by flow cytometry for DNA content. Nuclei, rather than whole cells, were used for the analysis, because cell aggregation prohibited flow cytometry analysis of cells. (A) Flow cytometry histograms of nuclei from cells cultured for 0 and 8 d in the absence or presence of NGF. Because the nuclear envelope of eukaryotic cells breaks down during M phase (Murray and Hunt, 1993), we have assumed that the 4n G2/M readings reflect the percentage of G2 nuclei and that M phase cells were lost from the population. (B) The percentage of G0/G1 phase (2n), S phase (~3n), and G2 phase (4n) nuclei are shown for both untreated and NGF treated cells at each time point. This analysis has been repeated four times.

$p33^{cdk2}$ and $p34^{cdc2}$ decreases in PC12 cells after NGF treatment. We speculate that NGF leads to the down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ expression and activity during the development of sympathetic neurons and certain sensory neurons—those neurons requiring NGF for survival. Indeed, fully differentiated sympathetic neurons of the rat superior cervical ganglia do not express cdk2 and cdc2 mRNA, whereas the cells do express

other cdks (Freeman *et al.*, 1994). The down-regulation of these activators of cell cycling is most likely responsible for the postmitotic arrest of NGF-dependent neurons in vivo and is likely to be required for their survival.

We observed that the down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ kinases, as well as the cellular differentiation, was accelerated in PC12-related cells that overexpressed the $p140^{trk}$ receptor (Hempstead *et al.*, 1992). This suggested that the level of $p140^{trk}$ was rate-limiting for the NGF-induced down-regulation of $p33^{cdk2}$ and $p34^{cdc2}$ kinases. This raises the interesting possibility that the level of $p140^{trk}$ receptors on sympathetic and sensory neurons may determine the timing of proliferative arrest and differentiation during the development of the nervous system.

A decrease in the expression of the cdc2 gene has been observed during the development of the central nervous system. A decline in cdc2 mRNA or protein levels was observed during the terminal differentiation of neurons in the rat cerebral cortex, cerebellum, hippocampus, and olfactory bulb (Hayes *et al.*, 1991; Okano *et al.*, 1993). Although it is unlikely that NGF is regulating the proliferation of these CNS neurons, other neurotrophic factors may be responsible. These factors may behave similarly to NGF in down-regulating the expression of $p33^{cdk2}$ and $p34^{cdc2}$.

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