Nerve Growth factor regulation of cyclin D1 in PC12 cells through a p21RAS extracellular signal-regulated kinase pathway requires cooperative interactions between Sp1 and nuclear factor-kappaB.

Francesco Marampon  
*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University*

Mathew C Casimiro  
*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University*

Maofu Fu  
*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University*

Michael J Powell  
*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University*

Vladimir M Popov  
*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University*


https://jdc.jefferson.edu/cbfp/26
Nerve Growth Factor Regulation of Cyclin D1 in PC12 Cells through a p21RAS Extracellular Signal-regulated Kinase Pathway Requires Cooperative Interactions between Sp1 and Nuclear Factor-κB

Francesco Marampon,*† Mathew C. Casimiro,**♭ Maofu Fu,* Michael J. Powell,* Vladimir M. Popov,* Jaime Lindsay,* Bianca M. Zani,♭ Carmela Ciccarelli,♭ Genichi Watanabe,§ Richard J. Lee,§ and Richard G. Pestell*

*Department of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107; ‡Department of Experimental Medicine, University of L’Aquila, 67100 L’Aquila, Italy; and §Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY 10461

Submitted December 15, 2006; Revised March 6, 2008; Accepted March 19, 2008
Monitoring Editor: William Tansey

The PC12 pheochromocytoma cell line responds to nerve growth factor (NGF) by exiting from the cell cycle and differentiating to induce extending neurites. Cyclin D1 is an important regulator of G1/S phase cell cycle progression, and it is known to play a role in myocyte differentiation in cultured cells. Herein, NGF induced cyclin D1 promoter, mRNA, and protein expression via the p21RAS pathway. Antisense- or small interfering RNA to cyclin D1 abolished NGF-mediated neurite outgrowth, demonstrating the essential role of cyclin D1 in NGF-mediated differentiation. Expression vectors encoding mutants of the Ras/mitogen-activated protein kinase pathway, and chemical inhibitors, demonstrated NGF induction of cyclin D1 involved cooperative interactions of extracellular signal-regulated kinase, p38, and phosphatidylinositol 3-kinase pathways downstream of p21RAS. NGF induced the cyclin D1 promoter via Sp1, nuclear factor-κB, and cAMP-response element/activated transcription factor sites. NGF induction via Sp1 involved the formation of a Sp1/p50/p107 complex. Cyclin D1 induction by NGF governs differentiation and neurite outgrowth in PC12 cells.

INTRODUCTION

Cyclin D1 is a member of the cyclin-dependent kinase (Cdks) family of serine/threonine kinases that play a pivotal role in controlling progression through the cell cycle (Pines, 1991; Motokura and Arnold, 1993). As the regulatory subunits of the Cdks, cyclin D1 complexes with catalytic partners to phosphorylate several different “pocket” proteins, including the retinoblastoma protein pRB and the related proteins, p107 and p130 (Beijersbergen et al., 1995; Mayol et al., 1995; Weinberg, 1995). The role of cyclin D1 in cell cycle regulation is complex. Antisense and immunoneutralizing experiments have demonstrated that cyclin D1 is capable of promoting G1 phase progression (Baldin et al., 1993; Quelle et al., 1993; Resnitzky et al., 1994; Resnitzky and Reed, 1995), but overexpression of cyclin D1 inhibits replicative DNA synthesis, implying cyclin D1 conveys a G1/S phase checkpoint function (Pagano et al., 1994). Cyclin D1 blocks PCNA relocation to the nucleus and subsequent binding to the replication initiation complex, an event required for normal S phase entry (Pagano et al., 1994). We and others have shown cyclin D1 is expressed in the developing central nervous system (Shambaugh et al., 1994; Watanabe et al., 1998b) with cyclin D1 transcripts detected in the anterior forebrain, the posterior midbrain, in the developing somites, and in the neural tube (Stahl et al., 2006). Deletion of both alleles for the cyclin D1 gene in transgenic mice resulted in failure of normal retinal neural layer development and abnormalities of neural regeneration (Fantl et al., 1995; Sicinski et al., 1995b). These studies emphasized the importance of the cyclin D1 protein in neural cell function.

NGF plays an important role in growth, differentiation, and survival of sympathetic and sensory neurons, and it promotes neuronal differentiation of PC12 cells (Greene and Tischler, 1976; Levi-Montalcini, 1976; Chao, 1992). Cyclin D1 expression is induced in postmitotic neurons (Freeman et al., 1989) or to increase the proportion of cells in G2 without affecting the proportion of cells in G0/G1 (Buchkovich and Ziff, 1994). Activation of the tyrosine kinase (Trk)A receptor by NGF induces p21RAS and sequentially the activity of several distinct kinases, including the raf kinases (B-raf), mitogen-activated protein kinase kinase

This article was published online ahead of print in MBC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–12–1110) on March 26, 2008.

† These authors contributed equally to this work.

Address correspondence to: Richard G. Pestell (richard.pestell@jefferson.edu).
(MEK)1, extracellular signal-regulated kinases (ERKs), phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ, and ribosomal S6 kinases (p90RSK) (Thomas et al., 1992; Stephens et al., 1994). Activation of p21^RAS or overexpression of MEK induces neurite outgrowth in PC12 cells (Bar-Sagi and Feramisco, 1985; Guerrero et al., 1986; Fukuda et al., 1995), and dominant-negative p21^RAS expression vectors blocked NGF-induced neurite outgrowth (Szeberényi et al., 1990; Thomas et al., 1992; Wood et al., 1992; Cowley et al., 1994). Thus, p21^RAS is capable of inducing a cascade of signaling events that lead to PC12 differentiation; however, the mechanisms leading to exit from the cell cycle and differentiation remain to be elucidated.

Neuronal outgrowth mediated by NGF requires p21^RAS, and it is associated with activation of the serine threonine kinases, ERKs. ERKs are induced by dual phosphorylation on threonine and tyrosine residues by MEKs (Cobb and Goldsmith, 1995; Marshall, 1995). Overexpression of p21^RAS, p74^RAF, MEK, and ERK are capable of inducing neurite outgrowth in PC12 cells, although ERK activation by growth factors may not be sufficient for the induction of neurite outgrowth (Bar-Sagi and Feramisco, 1985; Guerrero et al., 1986; Szeberényi et al., 1990; Cowley et al., 1994; Jaiswal et al., 1994; Lange-Carter and Johnson, 1994; Vaillancourt et al., 1995). NGF induces a Ras-dependent pp90^RSK kinase, which phosphorylates cAMP response element-binding protein (CREB) (Ginty et al., 1994). CREB binding sites are required for full NGF response of the immediate early genes ziff268 (DeFranco et al., 1993), nur77 (Yoon and Lau, 1993), the c-fos promoter (Ginty et al., 1994), and the neuronal secretory protein VGF (Hawley et al., 1992). For the c-fos promoter, NGF induction requires cooperation between a CREB binding site and a transcription factor bound to the serum response element (SRE) (Ginty et al., 1994).

The molecular mechanisms governing the genomic responses that occur after the delayed early genes, preceding and concordant with differentiation and exit from the cell cycle are currently an area of considerable interest. In the studies described herein, the intracellular signaling pathways governing NGF induction of cyclin D1 expression was determined in PC12 cells. As cyclin D1 was induced by NGF, we assessed the effect of either ectopic overexpression or antisense cyclin D1 on NGF-mediated neurite outgrowth. Cyclin D1 is required for NGF-mediated neurite outgrowth in PC12 cells.

MATERIALS AND METHODS

Cell Cultures and Treatment
PC12 and PC12-DN-RAS (M-M17-26) cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% defined and supplemented calf serum, 5% equine serum (heat-inactivated; Invitrogen), 10% glutamine, and 100 mg/ml penicillin and streptomycin, in a humidified 37°C incubator. Cells were plated on poly-l-lysine–coated dishes at the density of 2 * 10^6 cells/10-cm dish and starved for 24 h with 1% equine serum before all experiments. NGF (2.5S, murine NGF; Promega, Madison, WI) was used at 50 ng/ml. The MEK inhibitor PD098059 at 30 mM was a gift from Dr. A Saltiel (Life Sciences Institute, University of Michigan, Ann Arbor, MI). The p38 inhibitor SB203580 at 10 mM was supplied by SmithKline Beecham (Worthing United Kingdom). Wortmannin (200 nM) was supplied by Calbiochem (San Diego, CA), and neomycin (Invitrogen) was reconstituted and stored as recommended by the manufacturer.

Determination of Neurite Extension
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 et al., 1994). NGF induces neurite outgrowth in PC12 cells, and it is associated with activation of the serine threonine kinases, ERKs. ERKs are induced by dual phosphorylation on threonine and tyrosine residues by MEKs (Cobb and Goldsmith, 1995; Marshall, 1995). Overexpression of p21^RAS, p74^RAF, MEK, and ERK are capable of inducing neurite outgrowth in PC12 cells, although ERK activation by growth factors may not be sufficient for the induction of neurite outgrowth (Bar-Sagi and Feramisco, 1985; Guerrero et al., 1986; Szeberényi et al., 1990; Cowley et al., 1994; Jaiswal et al., 1994; Lange-Carter and Johnson, 1994; Vaillancourt et al., 1995). NGF induces a Ras-dependent pp90^RSK kinase, which phosphorylates cAMP response element-binding protein (CREB) (Ginty et al., 1994). CREB binding sites are required for full NGF response of the immediate early genes ziff268 (DeFranco et al., 1993), nur77 (Yoon and Lau, 1993), the c-fos promoter (Ginty et al., 1994), and the neuronal secretory protein VGF (Hawley et al., 1992). For the c-fos promoter, NGF induction requires cooperation between a CREB binding site and a transcription factor bound to the serum response element (SRE) (Ginty et al., 1994).

The molecular mechanisms governing the genomic responses that occur after the delayed early genes, preceding and concordant with differentiation and exit from the cell cycle are currently an area of considerable interest. In the studies described herein, the intracellular signaling pathways governing NGF induction of cyclin D1 expression was determined in PC12 cells. As cyclin D1 was induced by NGF, we assessed the effect of either ectopic overexpression or antisense cyclin D1 on NGF-mediated neurite outgrowth. Cyclin D1 is required for NGF-mediated neurite outgrowth in PC12 cells.
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.

**Immunohistochemical Staining and Western Blotting**

Immunohistochemistry was performed using the cyclin D1 antibody DCS-6 (Novacastra Laboratories, Vector Laboratories, Newcastle upon Tyne, United Kingdom) to confirm the immunoblot analysis, cells were lysed and sonicated for 30 s in 2% SDS containing phosphatase and protease inhibitors. Protein concentration was assessed by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and equal amounts were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a nitrocellulose membrane by electroblotting. Immunoblotting were performed with the following antibodies: cyclin D1 (05–815) was provided by Upstate Biotechnology (Charlottesville, VA), α-tubulin (sc-5546), phospho-ERK (sc-7383), total-ERK (sc-154), nuclear factor-xB (NF-xB) (p50) (sc-1190), p107 (sc-318), Sp1 (sc-420), c-Myc (sc-042), c-Jun (sc-1694), c-Fos (sc-52), CREB (sc-27585), and α-tubulin (sc-8686) were from Santa Cruz Biotechnology. Peroxidase-conjugate antii-mouse or anti-rabbit immunoglobulin G (Santa Cruz Biotechnology) was used for enhanced chemiluminescence detection.

**Immunoprecipitation**

PC12 cells were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100) in the presence of protease inhibitors at 4°C for 30 min. Insoluble debris was removed by microcentrifugation at 11,000 × g for 5 min. Proteins were immunoprecipitated by incubation in the presence of specific antibodies for 1 h at 4°C, followed by adsorption on protein G-Sepharose (GE Healthcare, Chalfont St. Giles, United Kingdom), and subjected to further Western blot analyses.

**Construction of Plasmid Vectors**

The 5′ promoter luciferase reporter constructions derived from the human cyclin D1 genomic clone have been described previously (Albanese et al., 1995; Watanabe et al., 1996b). Several heterologous constructions were made in which cyclin D1 promoter enhancer sequences were linked to the thymidine kinase (TK) promoter (TK1pA3LUC) and include CD1(AP-1)TKLUC (the cyclin D1 activator protein [AP]-1 site from -963 to -940), CD1(E2FTKLUC (the cyclin D1 sequences from -156 to -133), and CD1(CRE)TKLUC (sequences from -66 to -40). The nur77 reporter (nur77LUC) encodes the NGF-responsive sequences of the nur77 promoter from -86 to +122 (-86wLUC) (Yoon and Lau, 1994). The c-fos promoter from -361 to +157, cloned by polymerase chain reaction (PCR) using oligodeoxynucleotide probes to the published sequence and human genomic DNA, was subcloned into the pA3LUC reporter. The 5′ oligo primer sequence was 5′-GCC GCG GCC CAC CAG CAG TTC CCG-3′, and the 3′ oligo sequence was 5′ AAG CCT GTG GCC GTT TAG GCA AAG 3′. The c-fos SRE sequence (-333 to -291) was linked to the minimal E1BATALUC to form SRE1TALUC. The c-Myc P1 and P2 promoters from -157 to +500 (DesJardins and Hay, 1993) were linked to the pA3LUC reporter and form P1P2mycLUC. The P2 promoter derived from +66mycCAT (DesJardins and Hay, 1993) was cloned into the pA3LUC reporter to form P2mycLUC. The cyclin A promoter construction (Henglein et al., 1994) and the c-fos-responsive reporter containing three AP-1 sites, p107LUC, were described previously (Pestell et al., 1994b; Pestell et al., 1995). The reporter (UAS)E1BATALUC consists of the (UAS)E1BATTATA sequences from Gal4CAT cloned into the reporter pA3LUC (Watanabe et al., 1996b).

**Expression Vectors**

The plasmids RSV Ras L61 (Leu 61) (oncogenic p21 RAS), RSV Ras L61 S186 (Leu 61, Ser 186), (encoding oncogenic p21 RAS without the membrane targeting motif), RSV Ras N17 (the dominant interfering p21 RAS, Ras Asn17), RSV MAPK (ERKmt) (Seth et al., 1994), and the c-Jun-responsive reporter containing three AP-1 sites from the adenovirus E2a 940, CD1(E2F)TKLUC the oligodeoxyribonucleotide containing the wild-type (Yee et al., 1987) and mutant E2F sites (Weintraub and Dean, 1992) from the adenovirus E2 promoter were synthesized as complementary strands and cloned into BamHI-restricted TK1pA3LUC to form E2FTKLUC (Watanabe et al., 1998a).

**Transfections and Infection**

PC12 cells were plated 24 h before the transfection at a density of 80,000 cells/cm². The formulation of lipid–DNA or –small interfering RNA (siRNA) complexes was prepared by diluting siRNA and Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The Lipofectamine 2000–siRNA or –DNA complexes were then gently layered on the cultured cells. After 24 h, the cells were split onto poly-l-lysine–coated dishes, and then they were treated with 50 ng/ml NGF after 24 h. siRNAs against rat cyclin D1, p107, and Sp1 were purchased from Qiagen (Valencia, CA), and p50-siRNA was purchased from Santa Cruz Biotechnology. The -1745CD1LUC reporter was introduced into exponentially growing PC12 cells by transfecting 2 µg of -1745CD1LUC and 400 ng of pCDNA3 (Invitrogen) per 100-mm plate using the calcium phosphate precipitation technique. Stable transformants were selected using Geneticin (Invitrogen). Resistant cells were subcloned, and the cell lines -1745CD1PC12.5 and -1745CD1PC12.8 were selected. The Ras N17 stable PC12 cell line M-M17-26 was described previously (Szeberenyi et al., 1996). Retroviral infection was conducted as described previously (Neumeister et al., 2003).

**Electrophoretic Mobility Gel Shift Assays (EMSA)**

EMSA using cellular nuclear extracts or in vitro-translated proteins were performed essentially as described previously (Pestell et al., 1994a; Albanese et al., 1995). For EMSA, 5–10 µg of nuclear extracts was used in binding buffer containing 20 mM HEPES, pH 7.4, 40 mM KCl, 1 µM MgCl₂, 0.1 mM EDTA, and 0.1% NP-40 to which 0.5 ng of γ-32P-labeled probe and either 50 µg/ml poly(dI-dC), or for the E2F gel-shifts, 2 µg of sonicated salmon sperm DNA, as competitor, was added. Supershifts were performed with antibodies to c-jun (sc-922X), ATF-2 antibody (sc-187), and a c-Fos antibody (sc-253X) purchased from Santa Cruz Biotechnology and a CREB antibody that is also

**Figure 2.** NGF inhibits proliferation and induces cell cycle arrest in a Ras-dependent manner. (A) Growth curve of PC12 and PC12 (M-M17-26) cells counted at the indicated times. The data represent the mean ± SEM from n = 3 experiments. (B) FACs analysis of PC12 and PC12 (M-M17-26) cells treated (+) and untreated (−) with 50 ng/ml NGF for 14 d.
immunoreactive with cAMP response element modulator (CREM) (HM93). The zif268 antibody was a gift from Dr. L. Lau of the Johns Hopkins University School of Medicine (Williams and Lau, 1993). The reaction products were separated on 4% polyacrylamide gel run in 0.25 x Tris-borate-EDTA at 4°C at 180 v for 2–3 h. The gels were dried and exposed to XAR5 (Kodak) radiographic film.

**Reverse Transcriptase (RT)-PCR**

Cyclin D1 mRNA expression was determined by a qualitative RT-PCR assay. Total RNA was obtained from the PC12 or PC12 DN-RAS (M-M17-26) cells by using the RNAeasy Plus mini kit (QIAGEN). RNA levels and purity were assessed. The quantitative RT-PCRs were done using the Access RT-PCR System (Promega). Primer sequences were as follows: cyclin D1 Fwd, 5'-TGGAGCCCCTGAAGAAGAG-3' and cyclin D1, Rwd 5'-AAGTGCTTGTTGTGCGGTCG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Fwd, 5'-ACGACCCCTTCATTGACCTC-3' and GAPDH Rwd, 5'-GGGGGGTAAAGCAGTTGG-3'. PCR products were analyzed on a 1.5% agarose gel.

**p42ERK p44ERK and SAPK Immune-Complex Assays**

Assays were performed as described previously (Pestell et al., 1996b) on cell extracts from PC12 cells treated with 20 ng/ml NGF or with vehicle. For assays, staphylococcal protein A-Sepharose beads were incubated with anti-mitogen-activated protein kinase (MAPK) antibody (C16) (Santa Cruz Biotechnology), anti-stress-activated protein kinase (SAPK) antibody for 1 h at 4°C. The antibody and beads were washed once with radioimmunoprecipitation assay (RIPA) buffer, and then they were incubated with cell lysates for 2 h at 4°C. The immunoprecipitates were washed with RIPA buffer once, with LiCl/0.1 M Tris base, pH 8.0, twice, and once in kinase buffer. The kinase reactions were performed at room temperature for 20 min in 30 ml of kinase buffer with 10 mCi of [γ-32P]ATP (3000 Ci/mmol, 1 Ci = 37 GBq) and 4 mg of myelin basic protein (MBP) or 4 mg of c-Jun fusion protein for SAPK assays. The samples were analyzed by SDS-PAGE upon termination of the reaction with Laemml buffer and boiling. The phosphorylation of MBP or c-Jun was quantified by densitometry using a Fuji Bio Imaging Analyzer BAS 2000 (Fuji Photo Film, Kanagawa, Japan).

**Chromatin Immunoprecipitation (ChIP) Assay**

Cells were cultured for 24 h without NGF or with NGF 50 ng/ml. Formaldehyde was added to the cells to a final concentration of 1%, and then the cells were incubated at 37°C for 10 min. Cells (5 × 10⁶) were collected, and ChIP assay was performed using the EZ-Magna Chip (Upstate Biotechnology, Lake Placid, NY) following the manufacturer’s instructions. The cell lysate was sonicated to generate DNA fragments with an average length of 200-1000 bp. Genomic DNA enriched by antibody against Sp1 (sc-420; Santa Cruz Biotech-

**Figure 3.** Ectopic expression of anti-sense or siRNA to cyclin D1 blocks NGF induction of neurite outgrowth in PC12 cells. (A) PC12 cells transfected with either control vector, cyclin D1 sense, cyclin D1 antisense, siRNA control, or siRNA cyclin D1 were either untreated (−) or treated (+) for 72 h with NGF, and they were subsequently photographed using a phase-contrast microscope. (B) The same cells were analyzed for neurite length. (C) Cell lysates from the cells presented in A were analyzed by cyclin D1 immunoblotting to verify knockdown and stimulation with NGF. (D) Growth curve of PC12 cells transfected with control vector, cyclin D1 sense, and cyclin D1 antisense for the indicated times.

Vol. 19, June 2008 2569
nology) was purified by phenol extraction. Immunoprecipitated DNAs were subjected to PCR amplification (35 cycles of 95°C for 20 s, 64°C for 20 s, and 72°C for 1 min) of the cyclin D1 promoter by using specific forward (5′-TTCTCTGGGGCCTTGATCTC-3′) and reverse (5′-CTCTCGCTAATGCCTAACA-3′) primers from Takasawa et al. (2000), to amplify the promoter region (-92 to +27 nucleotides in relation to the transcription start site). The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized after ethidium bromide staining. As a control for DNA content, PCR reactions were also performed on chromatin samples before immunoprecipitation.

**Cell Proliferation Assay and Fluorescence-activated Cell Sorting (FACS) Analysis**

PC12 cells were counted using a hemocytometer, and then tested for exclusion of trypan blue. Data are expressed as average of triplicate ± SEM. PC12 cells were harvested by trypsin-EDTA and washed; pellets were resuspended in 0.3 ml of 50% fetal calf serum in phosphate-buffered saline, treated with 0.9 ml of 70% ethanol, and left overnight in the dark at 4°C before FACS analysis (FACSCaliber; BD Biosciences, San Jose, CA) as described previously (Rao et al., 2007).

**RESULTS**

**NGF Induction of Cyclin D1 Requires p21RAS in PC12 Cells**

We first determined the concentration of NGF request to induce cyclin D1, based on the concentration known to induce PC12 cellular differentiation. Time course experiments were conducted using several concentrations of NGF (25, 50, or 75 ng/ml). Cyclin D1 was induced at each NGF concentration, and 50 ng/ml was used in subsequent analysis (Figure 1A). Immunohistochemical studies were performed using the cyclin D1-specific antibody to determine the effect of NGF on cyclin D1 abundance. Serum-starved PC12 cells were treated with NGF for 3 and 8 h. The abundance of cyclin D1 was low in serum-starved cells, and it was induced by NGF in most cells within 8 h (Figure 1B). In serum-replete conditions, the nuclear abundance of cyclin D1 varied between cells, consistent with previous observations that the nuclear abundance of cyclin D1 varies throughout the cell cycle. Treatment with NGF for 8 h induced nuclear staining for cyclin D1 in >95% of the cells (Figure 1B).

Because NGF regulates the TrkA/p21RAS pathway, we investigated whether NGF induced cyclin D1 expression via p21RAS. A time course experiment was conducted in presence or absence of 50 ng/ml NGF by using PC12 cells, and the PC12 dominant-negative Ras stable cell line M-M17-26 (Szeberényi et al., 1990). NGF induced cyclin D1 in PC12 cells and activated ERK, whereas the same treatment did not affect cyclin D1 abundance or ERK activation in the Ras dominant-negative PC12 (M-M17-26) (Figure 1C).

To determine the effect of NGF on growth and cell cycle distribution of PC12 and Ras dominant-negative PC12 cells

![Figure 4](http://example.com/figure4.png)

**Figure 4.** NGF stimulates cyclin D1 promoter activity. (A) Dose response for NGF (1–50 ng/ml) in PC12 cells stably transfected with the -1745 CD1LUC reporter. The data represent the mean ± SEM of n = 6 separate transfections. (B) Time course assessment of NGF induction of -1745 CD1LUC reporter activity. The mean data ± SEM of n = 6 separate transfections are shown. (C) NGF induction of -1745CD1Luc reporter promoter activity was assessed in PC12 cells or in the PC12 (M-M17-26) (D) The dominant-negative p21ras (Ras N17) or activating p21ras mutants (RasL61) were transfected into the -1745 CD1LUC PC12 cells. NGF induction of promoter activity was assessed following 12 h of stimulation (50 ng/ml). The p21ras mutant RasL61 is incapable of inserting in the plasma membrane. The mean data ± SEM of n = 5 separate transfections are shown. Data were normalized to the control (empty expression vector). (E) RT-PCR analysis performed on PC12 and PC12 (M-M17-26) cells, untreated (−) or treated (+) with 50 ng/ml NGF for indicated times by using specific primers for cyclin D1.
(M-M17-26), cellular proliferation and FACS analysis were conducted. NGF treatment induced growth arrest of PC12 cells, whereas proliferation of the Ras dominant-negative PC12 cells (M-M17-26) was unaffected by NGF treatment (Figure 2A). FACS analysis was conducted after 14 d of NGF treatment. NGF treatment increased the proportion of PC12 cells in the G0/G1 phase and reduced the number of cells in the G2/M phase, whereas the cell cycle distribution in the Ras dominant-negative PC12 cells (M-M17-26) was not affected (Figure 2B). Together, these results indicate that the activation of TrkA-p21RAS pathway by NGF is essential to induce cyclin D1 expression, growth arrest, and differentiation.

**Cyclin D1 Is Required for Full NGF-induced Neurite Outgrowth**

To study the functional significance of cyclin D1 in NGF-induced differentiation, we knocked down cyclin D1 and assessed neurite outgrowth. PC12 cells were infected with a cyclin D1 cDNA sense or antisense expression vector under control of the tetracycline-regulated promoter (Tet-Off), or they were transfected with cyclin D1 siRNA. Expression of cyclin D1 antisense efficiently reduced NGF-induced neurite outgrowth, whereas the overexpression of cyclin D1 alone did not affect neurite outgrowth irrespective of NGF stimulation (Figure 3, A and B). The relative length and number of neurites were also reduced in the cyclin D1 siRNA PC12-transfected cells treated with NGF (Figure 3, A and B). Immunoblotting analysis verified the transfection efficiency and the reduction in cyclin D1 abundance (Figure 3C). To determine the effects of cyclin D1 on PC12 cellular growth, proliferation analysis was conducted in PC12 cells infected with a cyclin D1 cDNA sense or antisense expression vector. Expression of cyclin D1 antisense reduced PC12 cellular proliferation. Thus, cyclin D1 is essential for the maintenance of PC12 cell proliferation potential, and it is essential for the NGF-induced neurite outgrowth. Cyclin D1 overexpression alone is not sufficient to induce differentiation.

**NGF Stimulates Cyclin D1 Promoter Activity and mRNA Synthesis through p21RAS**

Further studies were conducted to determine the mechanism by which NGF induces cyclin D1. A ~1745-base pair cyclin D1 promoter fragment linked to luciferase gene was transfected into PC12. The concentration of NGF required for maximal induction of the -1745CD1LUC reporter in PC12 cells was determined using increasing doses of NGF for 24 h. Maximal (6-fold) induction of the cyclin D1 promoter was observed with NGF at a concentration of 50 ng/ml (Figure 4A). A time course experiment demonstrated that the cyclin D1 promoter was induced maximally (3- to 6-fold) by NGF, and it peaked at 6 h (Figure 4B). To determine whether the NGF effects on endogenous cyclin D1 transcripts levels were dependent upon p21RAS, we used three different approaches. First, we analyzed the cyclin D1 promoter activation by NGF treatment in M-M17-26-PC12 cells. NGF-induced cyclin D1 promoter activity was reduced 70% in the presence of RasL61 (Figure 4D). Quantitative RT-PCRs assay of mRNA levels in PC12 and M-M17-26-PC12 cells treated or untreated with 50 ng/ml NGF demonstrated NGF induced cyclin D1 mRNA in the PC12 cells but not in the Ras dominant-negative PC12 cells (M-M17-26) (Figure 4E).

Previous studies demonstrated that NGF induced expression of several immediate early genes, including c-fos, nur77, and c-myc in PC12 cells (Greenberg et al., 1985). The effect of NGF on these genes promoters was assessed using PC12 and Ras dominant-negative PC12 cells (M-M17-26) transfected with the promoter of c-fos, nur77, c-myc, cyclin A, or pA3 linked to the luciferase gene. The c-fos promoter was stimulated fivefold at 6 h, the c-myc promoter was induced 4.5-fold, and the nur77 promoter reporter was induced fourfold (Figure 5A). In contrast, the plasmid pA3LUC and the cyclin A LUC reporter were not induced by NGF, indicating the induction of the cyclin D1 promoter by NGF was gene-dependent (Figure 5A). Compared with the wild type PC12 cells, the induction by 50 ng/ml NGF in the M-M17-26 cells was reduced for c-fosLUC from eightfold to twofold, and for c-mycLUC from fourfold to twofold, consistent with previous studies demonstrating the importance of the Ras pathway in NGF-induced c-fos promoter activity (Ginty et al., 1994). The induction of the nur77 promoter was maintained in the M-M17-26 cells (Figure 5A), consistent with previous observations in which the induction of nur77 mRNA was unchanged in M-M17-26 cells (Szeberényi et al., 1990). Because previous studies demonstrated a role for p21RAS in c-fos induction by NGF (Ginty et al., 1994), experiments were conducted with the c-fosLUC reporter. RasL61 induced the c-fosLUC reporter threefold and overexpression of RasN17 reduced NGF induced c-fosLUC activity 60% (data not shown). We next determined the effect of NGF on c-Myc,
c-Fos, and c-Jun protein expression. As show in Figure 5B, NGF induced a sustained expression of c-Fos, c-Jun, and CREB, a transient expression of c-Myc in PC12 cells, and no modulation of these proteins occurred in M-M17-26 cells.

**ERK, p38, and PI3K Pathways Are Required for Full NGF Induction of Cyclin D1**

Ras is known to mediate several different pathways in response to epidermal growth factor (EGF), including the ERKs, Raf-1, and the RSK kinases (Wood et al., 1992). To characterize the intracellular signaling pathways involved in NGF-induced cyclin D1 promoter activity, MAPK assays were performed on NGF-treated PC12 cells. NGF-induced ERK activity eightfold in 10 min, and ERK activity remained elevated at 2 h. ERK activity had returned to basal at 6 h (Figure 6A). SAPKs were induced fourfold at 10 min, as described previously (Szeberényi et al., 1990) (Figure 6B).

To investigate the role of the MEK/ERK, p38 and PI3K pathways in basal and NGF-induced expression of cyclin D1, -1745CD1LUC–transfected PC12 cells were treated with NGF either alone or with the addition of the MEK (PD98059), p38 (SB203580), or PI3K (wortmannin) inhibitors. Furthermore, PC12 cells were cotransfected with the dominant-negative mutants of MEK (MEK-C), ERK (MAPKi), or with SAPK/ERK kinase (SEK) dominant-negative mutants (SEKKR and SEKAL). Luciferase activity was assessed after 24 h of treatment. As shown in Figure 6C, both the MEK, p38, and PI3K inhibitors and MEK-C, or MAPKi reduced the NGF-induced cyclin D1 promoter activity 40% (PD98059), 68% (SB203580), 70% (wortmannin), 80% (dominant-negative MEK), and 50% (dominant-negative ERKs), respectively. Conversely, the cyclin D1 promoter activity was not affected by overexpression of the SEK dominant-negative mutants SEKKR or SEKAL.

Because p21RAS controls activation of the PI3K and ERK pathways, we investigated the role of these downstream pathways in NGF signaling to cyclin D1. PC12 cells transfected with the -1745CD1LUC reporter were treated with PD98059, wortmannin, or both in the presence of NGF, and then they were processed for luciferase assay and Western blot. Inhibition of the ERK or PI3K pathway reduced NGF-induced cyclin D1 promoter activity (Figure 6D). Simultaneous inhibition of ERK and PI3K pathways (PD98059 + wortmannin) increased this effect, further suggesting that these pathways independently contribute to NGF signaling to the cyclin D1 promoter (Figure 6D). Finally, we analyzed the effects of ERK, p38, and PI3K pathway inhibition on NGF induction of cyclin D1 protein abundance. Inhibition of the ERK, p38, or PI3K pathway strongly reduced cyclin D1 induction by NGF (Figure 6E). Interestingly, p38 and PI3K pathway inhibition reduced the basal ERK activation status and reduced ERK activation by NGF (Figure 6E, left). Together, these data demonstrate that cyclin D1 induction by NGF involves the ERK, p38, and PI3K pathways (Figure 6E, right).

**Cyclin D1 Promoter Sequences Involved in Basal and NGF Responsiveness**

To identify the promoter regions that are necessary for NGF-induced cyclin D1 expression, and to understand the mecha-
isms that regulate this expression, we generated a series of 5′ promoter deletion constructions linked to the luciferase gene. Transfected PC12 cells were examined for basal and NGF-regulated activity (Figure 7). Basal elements were identified between -963 and -420 (Figure 7A). Deletion of the promoter sequence from -1745 to -163 had no effect on NGF-induced promoter activity (Figure 7B), whereas deletion from -163 to -22 dramatically reduced NGF-induced promoter activity (Figure 7B), suggesting that sequence within the proximal -163 base pairs of the cyclin D1 promoter were involved in NGF-induced activity.

Several promoter elements have been identified within the proximal -163 base pairs of the cyclin D1 promoter capable of mediating a response to NGF, including an E2F site (-147), an Sp1 site (-130), an Egr1 (-117), an LEF (-82) (Figure 7C), an ATF2 site (-58), or the NF-κB site (-39) (Figure 7D). Mutation or deletion of the E2F site (-147), Sp1 (-130) (Figure 7D), ATF2 site (-58), and NF-κB site (-39) (Figure 7D) reduced NGF-mediated cyclin D1 promoter activity. Together, these findings indicate that several sequences within the context of the native promoter may contribute to NGF responsiveness, including the E2F, Sp1, NF-κB, and the cAMP-response element/activated transcription factor (CRE/ATF) site.

NGF Induces the Sp1/p107/p50 Transcription Complex in PC12 Cells

To characterize the complex binding to the cyclin D1 cis elements required for full NGF responsiveness, EMSA was performed with untreated or NGF-treated PC12 cell nuclear extracts. Consistent with the cyclin D1 reporter assay, the cyclin D1 ATF/CRE site bound a complex in response to NGF treatment in a time-dependent manner (Figure 8A). We analyzed E2F binding to the cyclin D1 promoter, but the relative abundance of E2F complexes was not significantly altered by NGF at 12 and 24 h (Figure 8B). NGF can mediate induction of cyclin D1 via transcription factor Sp1 (Yan and Ziff, 1997); therefore, further studies of the cyclin D1 Sp1 site were conducted. ChIP assay performed in PC12 cells either untreated, or treated with NGF, showed that NGF induced a strong recruitment of Sp1 to the cyclin D1 promoter (Figure 8C). Sp1 knockdown experiments, conducted in PC12 cells, reduced Sp1 abundance and reduced cyclin D1 induction by NGF, confirming the functional significance of Sp1 in NGF-induced cyclin D1 expression (Figure 8D). PC12 cells, processed for immunoprecipitation with Sp1 antibody, were subsequently immunoblotted for p107 and p50. Western blot analyses demonstrated that p107 and p50 associate with Sp1. Furthermore, NGF increased the association of p50 and p107 with Sp1 (Figure 8F). Together, these findings demonstrate p107 and p50 associate with Sp1 and that NGF increases p50 and p107 association with Sp1.

NGF Induces Cyclin D1 Promoter Activation and Protein Expression Through a Sp1/p107/p50 Transcription Complex

Because our studies indicated that NGF treatment of PC12 cells increased the abundance of p107 and p50 to the Sp1 complex, we investigated the role of p107, p50, and Sp1 in NGF induction of cyclin D1 expression. p50 or p107 siRNA knockdown in PC12 treated with NGF reduced NGF induction of cyclin D1 (Figure 9A).

To examine whether p107 or p50 are recruited to Sp1, a mammalian two-hybrid analysis was conducted. An expres-
p50 and the Rb family member p107, under the control of E1A binding-defective p107 mutant (Watanabe et al., 1996). Expression of p107 increased Sp1 activity by 1.3-fold.

Expression vector encoding Gal4-Sp1 was cotransfected with expression vectors encoding either p107 or p50, together with a heterologous reporter construct containing 5X Gal4 DNA binding sites linked to a luciferase reporter gene into PC12 cells. Expression of p107 increased Sp1 activity by 1.3-fold. NGF treatment induced Sp1 activity threefold, whereas the E1A binding-defective p107 mutant (Watanabe et al., 1998a) failed to regulate Sp1 transactivation (Figure 9C). p50 enhanced NGF-induced Sp1 activity (Figure 9D). NGF increased p50, but not p107 expression, in a PI3K, ERK and p38-dependent manner (Figure 10). Thus, the NF-κB protein p50 and the Rb family member p107, under the control of p21RAS-dependent pathways, enhance the functional interaction with Sp1.

**DISCUSSION**

The current study demonstrates that NGF induction of cyclin D1 is required for neurite outgrowth in PC12 cells. Analysis of the molecular mechanisms by which NGF induces cyclin D1 has identified important roles for Sp1, NF-κB, and p107. Furthermore, these studies present a new mechanism by which NGF recruits p107 and the NF-κB protein p50 to the Sp1 protein to regulate cyclin D1 expression. In PC12 cells, cyclin D1 is induced by NGF, in a p21RAS-dependent manner, with delayed early kinetics. p21RAS was required for induction of cyclin D1 both in transient expression studies and in the stable RasN17 cell line M-M17-26. In contrast to cyclin D1, the cyclin A promoter was not induced by NGF, consistent with previous studies of cyclin A (Buchkovich and Ziff, 1994). Our studies indicate that these two cyclins are subjected to distinct control mechanisms during PC12 differentiation. NGF induction of cyclin D1 promoter activity and protein expression was inhibited by PD98059, SB203580, wortmannin, or overexpression of dominant-negative mutants of MEK or ERK but not SEK. Together, these studies demonstrate that NGF induction of the cyclin D1 promoter involves the p21RAS pathway.

Homozygous deletion of the cyclin D1 gene resulted in failure of normal retinal development (Fantl et al., 1995; Sicinski et al., 1995b), and these animals demonstrated abnormal neural reflexes, together suggesting a role for cyclin D1 in normal neural development. Herein, either cyclin D1 antisense or siRNA reduced NGF-induced neurite outgrowth in PC12 cells, demonstrating a requirement for cyclin D1 in cellular differentiation. Cyclin D1 regulates differentiation of myocytes (Skapek et al., 1996) and pregnancy-associated mammary epithelial cell differentiation (Sicinski et al., 1995a), suggesting a cellular context specific differentiation function. In cell type in which the cyclin D1/pRb pathway is inactivated by large T antigen, cyclin D1 did not contribute to neurite outgrowth, a function mediated by p35/CDK5 (Xiong et al., 1997).

Cyclin D1 was shown to be induced concordantly with NGF-induced neurite outgrowth in PC12 cells (Yan and Ziff, 1995); however, the molecular mechanisms involved were previously unknown. In previous studies examining cyclin D1 expression by proliferative stimuli, basal cyclin D1 expression was strongly reduced by chemical inhibitors of the ERK (PD098059), p38 (SB203580) and PI3K (wortmannin) pathways, suggesting that p21RAS controls cyclin D1 expression via ERK, PI3K and p38. Overexpression of activating ERK mutants induce neurite outgrowth (Fukuda et al., 1995), the inhibition of p38 blocks NGF-induced neurite outgrowth (Morooka and Nishida, 1998) and the PI3K/Akt pathway plays a positive role in PC12 differentiation (Kim et al., 2004). We have therefore hypothesized that each of these pathway may contribute to NGF induction of cyclin D1 expression and thereby neurite outgrowth. Our studies with chemical inhibitors demonstrate that p38 inhibition and PI3K inhibition reduced ERK activation and cyclin D1 expression induced by NGF. These studies suggest the action of p38 and PI3K pathways may function through ERK activation.

The genetic response associated with the introduction of NGF involves sequential induction of immediate early genes, including c-fos, c-jun (AP-1, AP-1 proteins), and the orphan nuclear receptor nur77 (Greenberg et al., 1985; Wu et al., 1989; Yoon and Lau, 1993). A second group of delayed
early genes, including the tyrosine hydroxylase gene, is activated 1–2 h after NGF treatment. This activation is likely contributed to by the prior induction of AP-1 proteins (Gizang-Ginsberg and Ziff, 1994). A third class of “late genes” become active 18 h after NGF treatment, and they include genes such as neuron-specific intermediate filament protein, peripherin, tau-1 and the microtubule-associated proteins (microtubule-associated protein-1), the expression of which is enhanced with neurite extension (Drubin et al., 1985). In the current studies, multiple different sequences were involved in regulating the cyclin D1 promoter, whereas the relative abundance of transcription factor complexes binding to these sequences was increased in response to NGF treatment. NGF promoted the recruitment of p107 and the NF-B member p50 to an Sp1 complex. NGF enhanced Sp1 transactivation, whereas mutation of the CRE/ATF site reduced induction by NGF. CREB/CREM complexes were shown to bind the -58 sequences in electrophoretic mobility shift assays performed with PC12 cell nuclear extracts. The p90RSK pathway induces phosphorylation and activation of CREB, CREM is required for induction of c-Fos in response to NGF in PC12 cells (Ginty et al., 1994). Thus, NGF may induce the cyclin D1 promoter in a p90RSK-dependent manner through collaborative interactions between several different transcription factors which are induced by p90RSK.

Although cyclin D1 functions as a multimeric holoenzyme to phosphorylate and inactivate pRB, it possesses additional roles. For example, inactivation of the helix-turn-helix (HLH) protein MyoD occurs independently of pRB phosphorylation (Skapek et al., 1996). Cyclin D1 has also been shown to bind additional proteins including histone deacetylases (HDACs), nuclear receptors, and transcription factors (Hirai and Sherr, 1996; Fu et al., 2004). Cyclin D1 inhibits peroxisome proliferator-activated receptor γ-mediated adipocyte differentiation through regulation of histone acetyltransferase and histone deacetylase activity in a CDK-independent manner (Fu et al., 2005a,b), and it governs heterotypic signals that modulate cellular migration (Neumeister et al., 2003; Li et al., 2006). NGF-induced cyclin D1 expression was not associated with an induction in cyclin D1-immune kinase activity (Buchkovich and Ziff, 1994) (Pestell and Watanabe, unpublished), suggesting that the effect of cyclin D1 during differentiation of PC12 cells may, as in myocytes and adipocytes, occur in a pRB-independent manner. Myocyte differentiation involves a complex interplay between the abundance of cyclins/ cyclin-dependent kinase inhibitors (CDKIs) (Halevy et al., 1995; Parker et al., 1995; Skapek et al., 1996), the abundance and activity of the E2F proteins (Shin et al., 1995), and the phosphorylation and inactivation of the HLH protein MyoD (Skapek et al., 1996). Cyclin D1 is capable of modulating several of these processes, because the activity of E2F-de-
dependent reporters is in part regulated by cyclin D1 and also MyoD activity is inhibited by cyclin D1 (Skapek et al., 1996).

The CDKI p21\textsuperscript{CIP1} is induced by NGF in PC12 cells after 8 d (Yan and Ziff, 1995). The induction of cyclin D1 abundance by NGF may be important in regulating downstream target genes involved in neurite outgrowth, such as the CDKIs, or other cyclins, such as p35, which play a role in cortical neuron outgrowth (Nikolic et al., 1996). NGF may also promote cyclin D1 association with other proteins, such as proliferating cell nuclear antigen, in turn inhibiting their activity and thereby promoting neurite outgrowth. Last, cyclin D1 induction by NGF may function to promote phosphorylation of other structural substrates, such as MAP2 or Tau, the normal functions of which are required for neurite outgrowth; however, the requirement of cyclin D1 for this activity remains to be determined.

ACKNOWLEDGMENTS

We are grateful to Drs. F. McCormick, H. Bujard, M. Gossen, B. Henglein (Northwestern University), R. Maurer (Oregon Health and Science University), E. Harlow (Massachusetts General Hospital Cancer Center), S. McDonald, S. Cook (Babraham Institute), G. Johnson (Lineberger Comprehensive Cancer Center), and R. Davis (University of Massachusetts Medical School) for expression and reporter plasmids; Dr. A. Saltiel for PD098059; and J. Christie (SmithKline Beecham) for SB-203580. We thank Dr. A. McNicol for the PC12 cell line. This work was supported in part by R01CA70896, R01CA75503, and R01CA86072 (to R.G.P.). This project is funded in part under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analysis, interpretations, or conclusions.

REFERENCES


Buckovich, K. J., and Ziff, E. B. (1994). Nerve growth factor regulates the expression and activity of p33cdc2 and p34cdc2 kinases in PC12 chromomyo-


Chao, M. V. (1992). Neurotrophin receptors: a window into neuronal differ-

etiation. Neuron 9, 583–593.


Conrad, K. E., Oberwetter, J. M., Vaillancourt, R., Johnson, G. L., and Gut-


ras signalling pathway regulating pittuiary cell-specific gene expression. Mol.


MAP kinase kinase is necessary and sufficient for PC12 differentiation and


growth factor induces NGF1A through complex regulatory elements that are

also sensitive to serum and phorbol 12-myristate 13-acetate. Mol. Endocrinol.

7, 365–379.


finger proteins control the absolute and relative activities of the two principal


Pestell, R. G. (2005a). Cyclin D1 inhibits PPAR

8

Fu, M., Rao, M., Bouras, T., Wang, C., Wu, K., Zhang, X., Li, Z., Yao, T.-P., and


cyclin D1 are small and slow detects in eye and mammary gland
development. Genes Dev. 9, 2364–2372.

Freeman, R. S., Estus, S., and Johnson, E. J. (1994). Analysis of cell-cycle

related gene expression in postmitotic neurons: selective induction of cyclin


Fu, M., Rao, M., Bouras, T., Wang, C., Wu, K., Zhang, X., Li, Z., Yao, T.-P., and

Pestell, R. G. (2005a). Cyclin D1 inhibits PPAR-

2

28


mouse and other terminally differentiating cells. Science 267, 1024–1027.


Self-contained, tetracycline-regulated retroviral vector system for gene deliv-


Transcription of the human choricron gonadotropin a and b genes is negatively


Pestell, R. G., Albanese, C., Lee, R. J., Watanabe, G., Moran, E., Johnson, J., and

Jameson, J. L. (1996a). A potential role for cell cycle control proteins in

regulation of the cyclic adenosine 5

-adenosine 5'-monophosphate-responsive glycoprotein


Pestell, R. G., Albanese, C., Watanabe, G., Johnson, J., Ekuld, N., Lastoviecki, P., and

Jameson, J. L. (1995). Epidermal growth factor and c-Jun act via a common DNA regulatory element to stimulate transcription of the


Chem. 270, 18301–18308.

Pestell, R. G., Albanese, C., Watanabe, G., Lee, R. J., Lastoviecki, P., Zom, L.,


cleavage enzyme (CYP11A1) promoter by ras- and Ets-2-signaling pathways.


represses transcription of the human chorionic gonadotropin alpha and beta

genes through distinct types of CREs. J. Biol. Chem. 269, 31090–31096.


M. F., and Sherr, C. J. (1993). Overexpression of mouse D-type cyclins accel-

eralates G1 phase in rodent fibroblasts. Genes Dev. 7, 11781–11785.


