Mediation of Nerve Growth Factor-driven Cell Cycle Arrest in PC12 Cells by p53

SIMULTANEOUS DIFFERENTIATION AND PROLIFERATION SUBSEQUENT TO p53 FUNCTIONAL INACTIVATION

Upon stimulation with nerve growth factor (NGF), PC12 cells extend neurites and cease to proliferate by influencing cell cycle proteins. Previous studies have shown that neuritogenesis and a block at the G1/S checkpoint correlate with the nuclear translocation of and an increase in the p53 tumor suppressor protein. This study was designed to determine if p53 plays a direct role in mediating NGF-driven G1 arrest. A retroviral vector that overexpresses a temperature-sensitive p53 mutant protein (p53ts) was used to extinguish the function of endogenous p53 in PC12 cells in a dominant-negative manner at the nonpermissive temperature. NGF treatment led to transactivation of a p53 response element in a luciferase reporter construct in PC12 cells, whereas this response to NGF was absent in PC12(p53ts) cells at the nonpermissive temperature. With p53 functionally inactivated, NGF failed to activate growth arrest, as measured by bromodeoxyuridine incorporation, and also failed to induce p21/WAF1 expression, as measured by Western blotting. Since neurite outgrowth proceeded unharmed, 50% of the cells simultaneously demonstrated neurite morphology and were in S phase. Both PC12 cells expressing SV40 T antigen and PC12 cells treated with p53 antisense oligonucleotides continued through the cell cycle, confirming the dependence of the NGF growth arrest signal on a p53 pathway. Activation of Ras in a dexamethasone-inducible PC12 cell line (GSRas1) also caused p53 nuclear translocation and growth arrest. Therefore, wild-type p53 is indispensable in mediating the NGF antiproliferative signal through the Ras/MAPK pathway that regulates the cell cycle of PC12 cells.

NGF, a neurotrophic polypeptide, belongs to a closely related family of neurotrophins composed of brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5. These paracrine hormones activate the development, maintenance, and regeneration of neurons in the nervous system (1). NGF signals the development of sympathetic, sensory, and a population of central nervous system neurons through its high affinity receptor, TrkA.

The induction of neuronal differentiation invokes two interrelated cellular processes: progression through the stages of neurite outgrowth and cell cycle arrest (2). The rat pheochromocytoma cell line PC12, derived from a transplantable chromaffin tumor, provides a model system for the NGF-mediated conversion to a neuronal phenotype (3). PC12 cells contain both the tyrosine kinase (TrkA) and low affinity (p75NT) NGF receptors (4, 5). Differentiation requires the TrkA receptor and proceeds through the Ras/MAPK pathway (6, 7). NGF decreases the growth rate of PC12 cells (8) and, in the short term, causes synchronized PC12 cells to accumulate in the G1 phase of the cell cycle with a decrease in DNA synthesis (9). Continued exposure to NGF arrests the population in G1 with an increased number in the G2/M phase also (10). Long-term treatment of PC12 cells with NGF promotes terminal differentiation, in which the PC12 cells resemble sympathetic neurons with a cessation of division, increased substratum adherence, neurite extension, and catecholamine synthesis (3).

The tumor suppressor protein p53 is a DNA-binding phosphoprotein that helps regulate the cell cycle (reviewed in Ref. 11). Overexpression of wild-type p53 causes either G1 cycle arrest (12) or apoptosis (2). Inactivation of p53 is a common event in the development of malignancy and occurs in >50% of all human tumors (13). Transgenic mice that have the p53 gene disrupted develop normally (14), indicating the dispensability of these genes in normal survival and development. Furthermore, sympathetic and sensory neurons from p53 null mice can survive in the presence of neurotrophins (15). More significantly, however, the neuronal precursors in p53 knockout mice show an enhanced proliferative potential (16), supporting a specific role for p53 in mediating an antiproliferative signal to neurons. These experiments implicate the role of p53 and other cell cycle regulators in NGF-mediated growth arrest of neurons and neuronal progenitors.

The NGF-mediated cell cycle arrest of PC12 cells is concomitant with the nuclear translocation of p53 in PC12 cells and primary hippocampal neuronal cultures (17, 18). The importance of this subcellular movement of p53 was also shown by the stable expression of a p53 dominant-negative miniprotein, in which the cytoplasmic sequestration of wild-type p53 correlate...
lated with an inhibition of both PC12 cell and oligodendrocyte differentiation (17). Progression through the cell cycle has been shown to be governed by the family of cyclin-dependent kinases, their regulatory subunits (the cyclins), and a family of protein inhibitors (19). In particular, the cyclin-dependent kinase inhibitor p21/WAF1 (20) is a direct transcriptional target gene of p53 and plays an important role in p53-dependent growth arrest (21, 22).

In this study, we investigated the role of p53 in mediating the NGF antigogenic signal that regulates the cell cycle of PC12 cells. Experiments with PC12 cell lines overexpressing a temperature-sensitive p53 mutant protein (A135V; p53ts) showed that the functional inactivation of p53 undermines NGF-activated cell cycle arrest, whereas neurite outgrowth continues uninhibited. Our results suggest that the closely coupled processes of cell cycle arrest and neuritogenesis share overlapping regulators; however, the wild-type p53 protein is a key coordinator of the NGF-stimulated G1/S phase cell cycle checkpoint in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines**—PC12 cells and the mutant cell line PC12nm5 (from Dr. Lloyd Greene) were grown and maintained in complete Dulbecco’s modified Eagle’s medium as described (3, 23). Exponentially growing populations of PC12 cells were split and grown on collagen (Vitrogen), poly-l-lysine (Sigma), or poly-l-ornithine (Sigma) plates or coverslips at least 18 h before treatment with NGF (50 ng/ml), epidermal growth factor (EGF; 50 ng/ml), or basic fibroblast growth factor (bFGF; 50 ng/ml) in complete medium. Mouse NGF (β-subunit) was prepared and purified from mouse submaxillary gland as described previously (24). EGF and bFGF were purchased from Sigma.

**Immunocytochemistry with Conformation-specific Anti-p53 Monoclonal Antibodies**—In the immunocytochemical studies, PC12 cells were grown on poly-l-lysine or poly-l-ornithine coverslips and treated with NGF for 6 days, washed with phosphate-buffered saline (PBS), and rapidly fixed in a 20 °C mixture of methanol/acetone or 100% methanol. Naive PC12 cells were analyzed with the agarose overlay method (25), which allows for enhanced visualization of cellular components in round-shaped, blast-like cells. PC12 cells without the agarose overlay produced the same results, but yielded inferior images. For the agarose overlay method, the coverslip with undifferentiated PC12 cells was overlaid with a thin sheet of agarose, fixed in methanol/acetone, permeabilized in 0.5% Nonidet P-40, and incubated with anti-p53 monoclonal antibodies for 2 h at 37 °C. Differenntiated PC12 cells were fixed by the same procedure, but without agarose overlay, due to neurite shearing effects.

For immunofluorescent visualization, primary antibody staining was followed by staining with goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). Immunofluorescence microscopical visualization was performed with a murine avidin-biotin-peroxidase complex kit (Oncogene Science Inc.) and diaminobenzidine (Sigma) according to the manufacturers’ specifications. Cells were then studied and photographed with a Nikon camera using a Zeiss microscope or a Nikon Diaphot TMD microscope.

**Western Blotting**—For all Western blots, cells were harvested, washed, and stored frozen until all time points were collected. Cell lysates were prepared for Western blotting by homogenizing cells with a Dounce homogenizer or sonicator in lysis buffer containing 25 mM Tris, 2.5 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5 units/ml aprotinin, and 1 μg/ml leupeptin (26). For whole cell lysates, protein concentration was quantitated with the BCA reagent (Pierce) or Bradford reagent (Bio-Rad), and equal amounts of total protein were loaded onto SDS-polyacrylamide gel lanes. For the nuclear localization studies, lysates were fractionated into nuclear and cytoplasmic fractions (27); nuclear pellets were washed at least twice to remove all traces of the cytoplasmic fraction; nuclei were solubilized in sample buffer; and an equal number of nuclei were loaded per lane for SDS-polyacrylamide electrophoresis (18). After electrophoresis, gels were blotted onto nitrocellulose (Schleicher & Schuell) or Immobilon (Bio-Rad), and membranes were blocked with 10% nonfat milk dry milk for 1 h to overnight. Primary antibody incubation was performed in 5% nonfat dry milk or 3% bovine serum albumin in PBS with the mouse anti-p53 monoclonal antibody PAb421 (Oncogene Science Inc. or Dr. Arnold Levine) or the rabbit anti-p21/WAF1 antibody cg-397 (Santa Cruz Biotechnology). Washing with PBS containing 0.1% Tween 20 was performed between all subsequent steps. Primary antibody staining was followed by staining with horseradish peroxidase-conjugated horse anti-mouse IgG secondary antibody (Bio-Rad) or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Kirkegaard & Perry Laboratories) in PBS. All Western blots were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech) and Fuji XR film. All experiments were repeated three times with similar results. Lanes were scanned in an Amersham Pharmacia Biotech laser densitometer and/or a Bio-Rad Gel Doc 1000 to estimate relative levels (18).

**Overexpression of p53ts or SV40 Large T Antigen (Tag) in PC12 Cells**—The PC12 cell line was a gift from Dr. Arnold Levine) or the rabbit anti-p21/WAF1 antibody cg-397 (Santa Cruz Biotechnology). Washing with PBS containing 0.1% Tween 20 was performed between all subsequent steps. Primary antibody staining was followed by staining with horseradish peroxidase-conjugated horse anti-mouse IgG secondary antibody (Bio-Rad) or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Kirkegaard & Perry Laboratories) in PBS. All Western blots were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech) and Fuji XR film. All experiments were repeated three times with similar results. Lanes were scanned in an Amersham Pharmacia Biotech laser densitometer and/or a Bio-Rad Gel Doc 1000 to estimate relative levels (18).

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**Overview of the p53ts or SV40 Large T Antigen (Tag) in PC12 Cells**—The PC12 cell line was a gift from Dr. Arnold Levine) or the rabbit anti-p21/WAF1 antibody cg-397 (Santa Cruz Biotechnology). Washing with PBS containing 0.1% Tween 20 was performed between all subsequent steps. Primary antibody staining was followed by staining with horseradish peroxidase-conjugated horse anti-mouse IgG secondary antibody (Bio-Rad) or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Kirkegaard & Perry Laboratories) in PBS. All Western blots were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech) and Fuji XR film. All experiments were repeated three times with similar results. Lanes were scanned in an Amersham Pharmacia Biotech laser densitometer and/or a Bio-Rad Gel Doc 1000 to estimate relative levels (18).
PC12 cells were plated on poly-L-ornithine-coated tissue culture plates at 60% confluency the day before transfection. Plasmids were packaged in LipofectAMINE or LipofectAMINE plus liposome vehicles and transfected according to the protocol of Life Technologies, Inc. for PC12 cells. Cells were then placed in medium containing serum with or without NGF. Cells were harvested 2–3 days later and lysed in RLB buffer (Promega). Total cell lysates were assayed colorimetrically with a β-galactosidase assay kit (Promega) and for luminescence with a luciferase assay kit (Promega). Luciferase data were normalized relative to overall transfection efficiency as determined by β-galactosidase expression, and data are presented as normalized luciferase units. All transfection experiments were performed three times, with the assays in duplicate for each experiment.

Cell Cycle Arrest Assays—Morphological and cellular changes of PC12, PC12(vector), and PC12(p53ts) cells in response to NGF were examined in conjunction with bromodeoxyuridine (BrdUrd) incorporation (10 μM, 1–2 h). BrdUrd labeling was analyzed by both immunocytochemistry and flow cytometry to concurrently measure cell cycle phase lengths and distributions. For immunocytochemical studies, cells were grown on poly-L-ornithine-coated coverslips, washed several times with PBS, fixed in a HCl/ethanol mixture, and stained with a horseradish peroxidase-conjugated anti-BrdUrd antibody (Roche Molecular Biochemicals) as described by the manufacturer. Cell immunostaining was visualized colorimetrically with dianisidine dihydrochloride following the protocol of the manufacturer, and then the cells were counterstained with 5 mg/ml of eosin Y (Sigma). Cells were observed and photographed using a Nikon Diaphot TMD microscope with a Nikon camera.

For flow cytometric studies, cells were washed with PBS, fixed with 70% ethanol, extracted with 3 N HCl to remove histones, and stained with a fluorescein-conjugated anti-BrdUrd antibody (Becton-Dickinson). Counterstaining with propidium iodide (Sigma) and single cell analysis were performed as described (29). A minimum of 10,000 cells/sample were analyzed using a Coulter Epics Elite ESP cytometer. A region was defined by a line drawn around the BrdUrd-positive cells and the percentages of S phase cells were quantitated using Coulter Epics Elite Multigraph software.

p53 Antisense Oligonucleotide Construction—A 20-base antisense oligonucleotide that corresponds to 10 bases of the 5′-region and 10 bases of the coding region of rat p53 (5′-TGT GAA TCC TCC ATG ACA GT-3′) was made (cf. Ref. 34). A corresponding sense oligodeoxynucleotide was used as a negative control. The antisense and sense oligonucleotides (Genosys Biotechnologies, Inc.) were synthesized with a thiol group at the 3′-end and with fluorescein conjugated at the 5′-end to enable visualization of the cellular uptake of the oligonucleotides by fluorescence microscopy (35, 36). The sulfhydryl groups of both oligonucleotides were coupled to penetratin I (Oncor, Inc.), a 16-residue antitumor peptide and localized to the nucleus in this time period, as viewed by fluorescence microscopy. NGF (50 ng/ml) was then added to one plate of each antisense or sense oligonucleotide-containing culture along with other appropriate controls.

RESULTS

Subcellular Localization of p53 upon NGF Activation of PC12 Cells—Since nuclear translocation of p53 has been reported to be an important part of its activation process (17, 18), we examined this process further. The p53 protein can exist in two different states (mutant/proliferative or wild-type/antiproliferative) that may differ in conformation or degree of phosphorylation (11, 39). Specific monoclonal antibodies (40, 41) that distinguish between these two conformational states of p53 under denaturing conditions were used to probe for subcellular localization by immunocytochemistry. The proliferative p53 form (recognized by PAb240) was localized to the cytoplasm alone (Fig. 1A), whereas the antiproliferative p53 state (recognized by PAb246) was present in the nucleus as well as the cytoplasm (Fig. 1B). Thus, we have demonstrated the presence of both conformational forms of p53 in normal PC12 cells with distinct subcellular localizations. Cells stained with the general PAb241 antibody, which binds both wild-type and mutant p53 proteins (42), showed a more intense immunoreactivity, positive in both compartments (Fig. 1C), similar to data obtained by others (17).

NGF-differentiated PC12 cells were treated for 6 days and immunostained for p53 forms. The proliferative anti-p53 antibody (PAb240) stained the cytoplasm only and not the nucleus (Fig. 1D), as in the naive PC12 cells. Both antiproliferative p53-specific PAb246 (Fig. 1E) and common binding PAb241 (Fig. 1F) antibodies again stained nuclear and cytoplasmic regions. All three monoclonal antibodies revealed qualitative increases in immunoreactive intensity upon differentiation. Negative immunostaining controls such as an unrelated primary antibody or secondary antibody conjugates in the absence of primary antibody produced no staining (data not shown). Hence, these data suggest that the proliferative mutant conformation of the p53 protein stays sequestered within the cytoplasm upon NGF induction, whereas the wild-type species enters the nucleus and mediates G1 phase arrest. Furthermore, these findings are consistent with previous Western blotting studies that demonstrated a quantitative increase in total and nuclear p53 proteins in PC12 cells after NGF activation (18).

Since cytoplasmic p53 levels are 5-fold higher than nuclear levels (18), the nuclear increase yielded only a small total cytoplasmic decrease that would easily escape detection by immunocytochemical techniques.

Specificity of the NGF Effect on p53—To test the specificity of NGF signaling through p53, other growth factors were examined for their effects on p53 protein levels. hFGF activates neurite outgrowth in PC12 cells, similar to NGF (43). Treatment of PC12 cells with hFGF for 1, 3, or 6 days caused a growth arrest, as seen with BrdUrd staining (data not shown), and an increase in p53 protein in the nucleus, similar to that caused by NGF (Fig. 2A). EGF affects many of the same signaling pathways in PC12 cells, but stimulates mitogenesis, not neuritogenesis (44, 45). EGF caused little or no increase in total
Fractions from PC12 cells after bFGF (1) or EGF (2) treatment. Cells were treated with 50 ng/ml growth factor; the nuclear fraction was prepared and electrophoresed; and the membrane was immunoblotted for p53 with anti-p53 PAb421. Densitometry of the bands indicated that the -fold increases for experimental lanes relative to each control were 2.4, 2.0, and 2.0 for NGF and 1.7, 2.0, and 1.9 for FGF at 1, 3, and 6 days, respectively (A). For EGF, the -fold increases were 1.2 and 0.9 at 1 and 2 days, respectively (B).

Overexpression of p53ts in PC12 Cells—The NGF induction of p53 protein expression is temporally correlated with cell cycle arrest in PC12 cells. To determine if a causal relationship exists between these two events, the endogenous wild-type form of p53 in PC12 cells was inactivated by the overexpression of a temperature-sensitive murine p53 mutant protein (p53ts). Exogenous p53ts acts in a dominant-negative fashion, oligomerizing with and inactivating endogenous p53 at the nonpermissive temperature. Upon switching to the permissive temperature, p53ts assumes the antiproliferative wild-type functional conformation. PC12 and PC12(vector) cells both exhibited a NGF-mediated concentration-dependent increase in XTT cleavage after 60 h (Fig. 3). This increased metabolic activity preceded cell cycle arrest, which occurred later by days 4–5 of NGF treatment. Based on the XTT profile, the PC12(p53ts) cells also responded to NGF in a concentration-dependent manner. However, these cells demonstrated enhanced NGF-dependent metabolic growth at 60 h compared with the PC12 and PC12(vector) cells (Fig. 3). Analysis at 72 h revealed a similar dependence of the XTT reaction on NGF concentration (data not shown).

K252a, an inhibitor of TrkA, inhibited metabolic activation by NGF in PC12(p53ts), PC12(vector), and PC12 cells at 1 μM with a maximal concentration of 100 ng/ml NGF as measured by XTT cleavage (data not shown). These results suggest that the increased sensitivity of PC12(p53ts) cells to NGF may be explained by TrkA receptor up-regulation. Alternatively, the functional inactivation of p53 by overexpression of p53ts at the nonpermissive temperature may lead to an augmentation of TrkA-stimulated intracellular signaling pathways.

Functional Inactivation of p53 in PC12(p53ts) Cells—Wild-type p53 functions by binding to p53 response elements and activating transcription of a wide array of genes (for review, see Ref. 11). In PC12 cells, the functional activity of endogenous wild-type p53 was monitored by its ability to transactivate a p53 response element. PC12 and PC12(p53ts) cells were transiently transfected with a luciferase reporter construct containing multiple consensus p53 response elements upstream of a hsp70 basal promoter. The p53 response element was based on sequences from several genes (48) (see “Experimental Proce-
and cotransfected with a p53 response element (p53 RE) luciferase reporter construct and cotransfected with a β-galactosidase reporter for normalization of transfection efficiency. After transfection, cells were placed in serum-containing medium with or without 2 nM NGF and harvested 2–3 days later. Total cell lysates were assayed colorimetrically for β-galactosidase and luciferase activities by scintillation counting. Luciferase data were normalized relative to overall transfection efficiency as determined by β-galactosidase expression, and data are presented as normalized luciferase units. All transfection experiments were performed in duplicate with assays in triplicate.

In the absence of NGF, PC12 cells demonstrated a very low level of p53 transactivation of the p53 response element/luciferase reporter, almost equal to that of the cells transfected with a plasmid containing only the basal promoter upstream of the reporter gene (Fig. 4). On the other hand, when stimulated with NGF, PC12 cells exhibited a high level of luciferase activity (Fig. 4). These data show that NGF-mediated differentiation of PC12 cells leads to the transcriptional transactivation of a p53 response element and are consistent with results from previous studies (17, 18).

In PC12(p53ts) cells at the nonpermissive temperature, NGF failed to elicit the transactivation response of the luciferase reporter (Fig. 4). These results demonstrate that exogenous p53ts is capable of blocking the functional activity of endogenous wild-type p53 in a dominant-negative fashion. Therefore, at the overexpressed level under the nonpermissive conditions, p53ts abrogates the NGF-mediated increase in endogenous p53 transcriptional activity.

Further immunocytochemical studies were performed to determine if p53ts inhibits NGF-induced nuclear localization of wild-type p53, as does a p53 dominant-negative miniprotein (17). In the presence of NGF, PC12(p53ts) cells displayed no nuclear immunoreactivity for a wild-type p53-specific monoclonal antibody (Fig. 5). These data demonstrate that p53ts sequesters wild-type p53 protein in the cytoplasm and prevents it from acting in the nucleus as a transcriptional modulator.

Inactivation of the Antiproliferative Effects of NGF on PC12(p53ts) Cells—The ability of NGF to promote growth arrest and to block DNA synthesis in the absence of functionally active p53 was measured by BrdUrd labeling. Any cell actively replicating DNA would have passed through the G1/S checkpoint and be considered to be proliferating. The cells were pulse-labeled, immunostained, and quantitatively analyzed by flow cytometry. This technique was used both to separate the G1, S, and G2/M populations and to directly measure positively immunoreactive S phase cells.

In PC12(p53ts) cells at the nonpermissive temperature in the absence of NGF, 33% of the cells were in S phase and did not undergo cell cycle arrest when stimulated by NGF after 4 days. In duplicate flow cytometric experiments, the percentage ± range of S phase cells in the control cells was 22 ± 3% without NGF and 11 ± 5% with NGF, whereas the PC12(Tag) cells showed 21 ± 7% without and 22 ± 6% with NGF. These PC12(Tag) cells still exhibited neuritogenesis upon addition of NGF, indicating unimpaired morphological differentiation (see below for comparison with PC12(p53ts) cells). Second, cells in
which p53 expression was blocked by treatment with antisense oligonucleotides directed against the translational initiation codon of p53 did not undergo cell cycle arrest after 4 days of NGF stimulation. In duplicate experiments, the percentage of S phase cells in the cells treated with sense oligonucleotides was 19 ± 1% without NGF and 10 ± 2% with NGF, whereas the cells treated with antisense oligonucleotides had 24 ± 3% without and 22 ± 2% with NGF in S phase. The induction of p21/WAF1 by NGF was blocked in PC12 cells treated with antisense oligonucleotides (data not shown), which is consistent with the functional inactivation of p53 and failure of cell cycle arrest. However, the specificity of this reagent in the inhibition of p53 expression is currently under investigation. These data with PC12(Tag) cells and p53 antisense oligonucleotides concur with findings in PC12(p53ts) cells and demonstrate convincingly that NGF requires p53 to exert its antiproliferative effects over the cell cycle arrest of PC12 cells.

**Neuritogenesis without Cell Cycle Arrest in PC12 Cells That Lack Active p53**—Preliminary experiments indicated that the PC12(p53ts) cells developed neurites in the presence of NGF. These neurites in both PC12 and PC12(p53ts) cells showed positive immunoreactivity and colocalization of neuromodulin with F-actin, indicating that the developing neuronal characteristics had not changed upon overexpression of p53. Immunocytochemical analysis of BrdUrd incorporation was employed to measure cell cycle status simultaneously with neurite outgrowth in the same cell. At the nonpermissive temperature, cells were pulse-labeled with BrdUrd, immunostained, and examined by light microscopy. Any cells undergoing neuritogenesis while in S phase would be detected as positive for neurites and have black nuclei. Naive PC12 cells were round and positive for BrdUrd incorporation (Fig. 7A). Similarly, PC12(p53ts) cells showed many immature cells undergoing DNA replication in the absence of NGF treatment (Fig. 7C). PC12 cells activated with NGF for 6 days extended neurites and exited the cell cycle as demonstrated by the paucity of positively immunoreactive nuclei (Fig. 7D). NGF elicited the expected pro-differentiative and antiproliferative responses from the PC12 cells. In comparison with the PC12(p53ts) cells, NGF stimulated neurite outgrowth without cell cycle arrest, which was demonstrated by the extensive population simultaneously containing both mature neurites and BrdUrd-immunoreactive nuclei (Fig. 7D).

These microscopy data are quantitated and shown in Fig. 7E. About 50% of the PC12(p53ts) cells showed both mature neu-
Western blot analysis of p21/WAF1 in PC12 and PC12(p53ts) cell lysates. Cells were incubated in the presence or absence of 50 ng/ml NGF for 4 days in serum-containing medium at the nonpermissive temperature. Western blotting was performed with an anti-p21/WAF1 antibody on 80 μg of protein from whole cell lysates that had been separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose.

|        | PC12 | PC12 [p53ts] |
|--------|------|-------------|
| p21/WAF1 → | -/+ | -/+ |
| relative density | 4.0 | 28.7 |

Fig. 8. Western blot analysis of p21/WAF1 protein in PC12 and PC12(p53ts) cell lysates. Cells were incubated in the presence or absence of 50 ng/ml NGF for 4 days in serum-containing medium with or without dexamethasone (DEX) for 1–4 days; the nuclear fraction was prepared and electrophoresed; and the membrane was immunoblotted for p53 with anti-p53 PAb421. The first, third, and fifth lanes are control untreated samples, and the second, fourth, and sixth lanes are treated samples. Densitometry of the bands indicated that the -fold increases for the dexamethasone-treated lanes relative to each control were 1.7, 3.5, and 2.3 at 1, 2, and 4 days, respectively.

Stable overexpression of p53ts was established to determine if a causal relationship existed between NGF-stimulated cell cycle arrest and p53 activation in PC12 cells. The PC12(p53ts) cells had an enhanced proliferative responsiveness at the nonpermissive temperature, at which p53ts is in its proliferative conformation (Fig. 3). A strong cell-surface and cell cycle-dependent expression of TrkA in the early G1 and M phases in PC12 cells has been reported (53). These studies suggest that the increased sensitivity of PC12(p53ts) cells to NGF may be explained by either TrkA receptor up-regulation or an augmentation of TrkA-activated intracellular signaling pathways preceding cell cycle arrest.

NGF increases p53 protein levels (18) and stimulates the transcriptional activity of p53 response elements (Fig. 4), events that temporally correlate with cell cycle arrest. In the PC12(p53ts) cells, the transcription-activating ability of endogenous p53 was abrogated at the nonpermissive temperature (Fig. 4). These results demonstrate that endogenous p53 is functionally inactivated by p53ts at the nonpermissive temperature even in the presence of NGF. NGF induction of PC12 cell differentiation is not accompanied by changes in transcription of the p53 gene (17), in contrast to differentiation of pre-B cells, which is associated with an up-regulation of p53 mRNA expression (54).

PC12(p53ts) cells were tested for their cell cycling capacity in the presence of NGF to determine if p53 was required in regulating the G1 phase cell cycle checkpoint. At the nonpermissive temperature, NGF did not inhibit the cell cycle progression of the PC12(p53ts) cells, whereas the normal PC12 cells were blocked in G1 phase at 6 days (Fig. 6). Note that the growth arrest was not seen in the XTT experiments at 48 h because the G1/S block only occurs after several days. PC12(p53ts) cells continued to extend neurites at the nonpermissive temperature (Fig. 7), thus producing individual cells that were in S phase with neurites (Fig. 7D). A failure in signaling cell cycle arrest while maintaining the capacity to differentiate may seem contradictory, but this paradox has been shown to exist in other cell systems (55–57). In addition, we blocked p53 function with Tag expression and with antisense oligonucleotides to p53 to confirm the essentiality of p53 for NGF-induced cell cycle arrest. Thus, p53 appears to be essential in NGF-driven antimitotic signaling in PC12 cells. These results support a model of two separate cellular pathways for cell cycle arrest and neuritogenesis with overlapping regulators.
Up-regulation of p21/WAF1 protein levels occurred in normal PC12 cells after 4 days of NGF treatment (Fig. 8), as described previously (18, 58). This response to NGF was absent in the PC12(p53ts) cells at the nonpermissive temperature (Fig. 8). In PC12(p53ts) cells, p21/WAF1 protein levels are suppressed; G1 cycle arrest is inhibited; and neuritogenesis proceeds unharmed. Other reports have also contributed to establish the significance of p53 and its transcriptional target, p21/WAF1, in mediating PC12 cell cycle arrest and neurite outgrowth. For example, inducible overexpression of p21/WAF1 in PC12 cells leads to permanent growth arrest without directly leading to differentiation (59). Also, the repression of NGF-induced neuritogenesis with a nitric-oxide synthase inhibitor correlates with a reduction of p53 protein levels that is restored by overexpression of p21/WAF1 (60). These observations support the hypothesis that p53 may control NGF-activated growth arrest at least in part through p21/WAF1.

In comparison, others have questioned the function of p53 in mediating p21/WAF1 expression during NGF stimulation of PC12 cells. Two endogenous p53 response elements upstream of the promoter region of p21/WAF1 were shown to be dispensable in NGF activation of a reporter construct (61). This p21/WAF1 5′-untranslated region contains two p53 response elements, but may lack critical enhancer elements farther upstream, intragenically, or downstream that facilitate p53 binding and that could provide an even higher level of trans-activation not detected by such experiments. Furthermore, the cooperation of p300, a large transcriptional coactivator, with Sp1 transcription factors in NGF-mediated p21/WAF1 gene regulation suggests the importance of enhancer elements that are most likely found outside of the central 5′-promoter region of p21/WAF1 (62). The p300 factor binds and synergizes with p53 transactivation (63), thus potentially arranging for a multi-protein complex with histone acetyltransferase activity and general transcription factors. Thus, these findings could be consistent with a role for p53 in mediating NGF-induced trans-activation of p21/WAF1 during cycle arrest and neuritogenesis.

This study demonstrates the requirement for a functional p53 protein in activating NGF-driven cell cycle arrest. NGF appears to regulate p53 nuclear translocation through the Ras/ MAPK signaling pathway (64), which results in p21/WAF1 transcription and accumulation of PC12 cells in the G1 phase of the cell cycle. For mediating neuritogenesis, the p53 protein has been reported both to be expendable (61) and to be required (17, 54, 60, 65). In particular, the capacity of PC12(p53ts) and PC12(Tag) cells for neurite outgrowth contradicts a report in which a p53 dominant-negative miniprotein (p53DD) inhibited NGF-mediated differentiation of PC12 cells (17). The p53 miniprotein lacks the entire N-terminal transactivation region and consists of minimal C-terminal residues that are competent for oligomerization, but not DNA binding. However, such truncated forms of the C terminus of p53 not only inhibit p53 response element transactivation, but also repress the transcription-activating domains of several other viral and cellular transcriptional activators (66). Alternatively, since the N terminus of p53 interacts with Mdm-2 and p300/CRBP (67), the sequestration of endogenous wild-type p53 with p53DD overexpression might affect the complex interactions of these transcription factors. The positive finding of our studies with two separate methods, showing that PC12 cells lacking transcriptionally active p53 could still undergo differentiation, would seem to override the earlier results (17, 54, 60, 65) and clearly demonstrates an uncoupling of differentiation from proliferation such that both may proceed under certain circumstances in PC12 cells (68). Our data indicate that the NGF inhibition of cell cycle progression is regulated in a p53-dependent manner, whereas neuritogenesis primarily, but not necessarily exclusively, relies on p33-independent mechanisms.

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Mediation of Nerve Growth Factor-driven Cell Cycle Arrest in PC12 Cells by p53: SIMULTANEOUS DIFFERENTIATION AND PROLIFERATION SUBSEQUENT TO p53 FUNCTIONAL INACTIVATION

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