Ectopic p21WAF1 Expression Induces Differentiation-specific Cell Cycle Changes in PC12 Cells Characteristic of Nerve Growth Factor Treatment*

(Received for publication, April 2, 1998, and in revised form, June 24, 1998)

Joseph A. Erhardt‡ and Randall N. Pittman
From the Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Nerve growth factor treatment of PC12 cells results in neuronal differentiation, a process accompanied by induction of the Cdk inhibitor p21WAF1. To determine the role of p21 in differentiation, PC12 clones containing an inducible p21 construct were utilized to induce growth arrest. Expression of p21 led to accumulation of cyclins D1 and E and to a decrease in cyclins A and B. Levels of Cdc2 and Cdk4 also decreased after p21 induction. Initially, thymidine incorporation into DNA was dramatically inhibited; however, low levels of incorporation were observed during prolonged p21 expression. Fluorescence-activated cell sorter analysis revealed that this low level of DNA synthesis resulted in the generation of polyploid cells. Results from Western blots were consistent with phosphorylation of p21 protein coincident with the resumption of DNA synthesis. Finally, treatment of p21-arrested populations with epidermal growth factor, a known PC12 mitogen, resulted in neurite extension, a key feature of neuronal differentiation. Overall, cell cycle changes following p21 overexpression in PC12 cells closely mimic distinctive events previously shown to occur during differentiation. These results suggest that the mechanism by which nerve growth factor induces the many cellular changes associated with growth arrest during differentiation is through p21WAF1 induction.

To understand aspects of neuronal differentiation, specifically to investigate events underlying cell cycle arrest during this process, the rat pheochromocytoma (PC12) cell culture model has frequently been employed (7). Undifferentiated PC12 cells proliferate in the presence of serum; however, addition of nerve growth factor (NGF) results in sympathetic-like neuronal differentiation, including complete mitotic arrest (7, 8). Using this system, certain distinctive cell cycle events have been shown to occur. During differentiation, these cells initially arrest in G1, although DNA synthesis continues at a low level, leading to an increase in the DNA content of individual cells (9, 10). At the protein level, cyclin D1 increases, cyclin B decreases, cyclin A decreases or remains unchanged, and cyclin E increases or remains unchanged. The cyclin-dependent kinases Cdc2, Cdk2, and Cdk4 have all been shown to decrease as these cells undergo proliferation arrest (11–14).

In addition to the above changes, the cyclin-dependent kinase inhibitor p21WAF1 increases at the protein level following NGF treatment of PC12 cells (11–14). p21 was initially isolated as a p53 response gene (15), and a protein increased during senescence (16) and differentiation (17). This gene product was also cloned based on interactions with other cell cycle components (18–20). p21 is known to directly inhibit kinase activities of a wide range of cyclin-Cdk complexes, resulting in a general arrest in the cell cycle (for review, see Ref. 21). Increased expression of p21 has been associated with differentiation of numerous cell types in addition to neurons, including myelocytes (22, 23), myoblasts (24, 25), and keratinocytes (26). Additionally, in vivo analysis of p21 expression reveals consistently high levels in numerous differentiated tissues, including brain (27, 28).

Given these data, an obvious potential mechanism underlying permanent withdrawal from the cell cycle during neuronal differentiation could be increased p21 expression. To study the role of p21 in this process, stable PC12 cell lines were generated containing an inducible p21 construct. In a previous report, this system was utilized to demonstrate that irreversible growth arrest occurs in these cells following induction of p21 (29). The aim of the present study was to investigate the possibility that p21 expression is responsible for the numerous characteristic cell cycle changes associated with terminal differentiation of PC12 cells following NGF treatment.

In this study, induction of p21 resulted in growth arrest accompanied by an increase in cyclin D1 and E expression. At the same time, a decrease in cyclins A and B, Cdc2, and Cdk4 was detected. Immediately after p21 induction, thymidine incorporation into DNA was almost completely inhibited; however, low levels of incorporation resumed during long-term

* This work was supported in part by National Institutes of Health Grant NS29465. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a grant from the Pharmaceutical Research and Manufacturing Association. To whom correspondence should be addressed: Dept. of Pharmacology, School of Medicine, University of Pennsylvania, 155 John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104. Tel.: 215-898-7099; Fax: 215-573-2236; E-mail: erhardt@pharm.med.upenn.edu.
growth arrest. FACS analysis revealed that this low level of incorporation was due to an increase in the DNA content of individual cells, rather than a resumption of proliferation. Western analysis of phosphatase-treated cell lysates suggested that p21 protein was phosphorylated during the time of resumed DNA synthesis; similarly, treatment of PC12 cells with NGF led to the same apparent modification. As a whole, these cell cycle changes are the same as those occurring during NGF-induced differentiation of PC12 cells, consistent with p21\(^{WAP1}\) playing a key role in this process.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture—**PC12 cell lines containing an inducible p21\(^{WAP1}\) construct were generated as described previously (29) using the Lac Switch inducible mammalian expression system (Stratagene, La Jolla, CA). A 3 mM concentration of the lactose analogue isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) was added to the culture medium to induce p21 expression, with maximal protein levels reached within 14–18 h. Cells were cultured in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 100 units/ml penicillin G, and 14–18 h. Cells were cultured in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 100 units/ml penicillin G, and 100 \(\mu\)g/ml streptomycin at 37 °C in 5% CO\(_2\). Clonal cell lines were cultured in the presence of 200 \(\mu\)g/ml hygromycin B and 300 \(\mu\)g/ml G418, to obtain selection. For differentiation, cells were subcultured onto tissue culture dishes precoated with 50 \(\mu\)g/ml rat tail collagen (Collaborative Biochemical, Bedford, MA) in the presence of 100 ng/ml EGF (Collaborative) in normal culture medium. Cells treated with 20 ng/ml epidermal growth factor (EGF) (Collaborative Biochemical) were also subcultured onto collagen-coated dishes in normal medium in the presence or absence of IPTG.

**Western Blots—**Total cellular proteins (50 \(\mu\)g/ml) were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to PolyScreen polyvinylidene difluoride transfer membrane (NEN Life Science Products). Antibodies employed to detect p21, cyclin D1, cyclin E, cyclin A, cyclin B, Cdk4, and Cdk2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For detection of p21, antibodies generated against the carboxyl-terminal 19 amino acids (p21(C)) or amino-terminal 20 amino acids (p21(N)) were utilized. Immunoreactive signals were detected with horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim) using Renaissance Western blot chemiluminescent reagent (NEN Life Science Products).

To detect potential phosphorylation events, cells containing p21 induced for 8 days were washed in phosphate-buffered saline and resuspended in lysis buffer containing 100 mM Tris (pH 8.0), 5 mM MgCl\(_2\), 100 mM NaCl, 0.1% Triton X-100, 10 \(\mu\)g/ml leupeptin, and 0.5 mM phenylmethylsulfonaryl fluoride. For lysates receiving no treatment, 2 \(\times\) Laemmli buffer was added immediately. Other lysates were incubated for 30 min at 30 °C with 0.2 units/\(\mu\)l alkaline phosphatase (Boehringer Mannheim) in the presence or absence of the phosphatase inhibitor \(\beta\)glycerophosphate (20 mM). Reactions were terminated by the addition of 2 \(\times\) Laemmli buffer.

**Measurement of Proliferation and Neurite Outgrowth—**Proliferation was assessed by counting the total number of cells in randomly selected fields after 0 days in the absence (control) or presence of 3 mM IPTG to induce p21 expression (six random fields/experiment). Over subsequent days, cells were counted in the same fields, and the relative number represents the ratio of cells on a given day to the number of cells at time 0. An average of 350 cells were counted at time 0 in five individual experiments.

Neurite extension was measured in randomly selected fields of PC12 cells plated on collagen. Proliferating and p21-arrested cells were cultured in the absence or presence of 20 ng/ml EGF. 4 days after EGF treatment, individual cells were scored based on the number of neurites extending >20 or 50 \(\mu\)m from the cell body. Approximately 60–90 cells/group were analyzed in each experiment, and the data represent the average of three experiments.

**Thymidine Incorporation—**Cells were treated in the presence or absence of IPTG to induce p21 expression for the indicated times and then incubated with 2 \(\mu\)Ci/ml \(^{3}H\)thymidine for 6 h. Plates were washed two times in cold phosphate-buffered saline and scraped into microcentrifuge tubes. A fraction of cells was removed for subsequent analysis of protein content. The remaining sample was incubated at 4 °C with 10% trichloroacetic acid, and precipitable material was collected on glass filters using a vacuum manifold apparatus. Relative thymidine incorporation into DNA was determined by scintillation counting.

**FACS Analysis—**Flow cytometric analysis of DNA content was essentially performed as described previously (10). Proliferating and p21-arrested populations were lysed in a buffer containing 0.3% Nonidet P-40, 5 mM NaCl, 10 mM trisodium citrate (pH 7.6), and 100 \(\mu\)g/ml DNase-free RNase A. Nuclei were isolated by low speed centrifugation, and the resulting pellet was resuspended in lysis buffer containing 5 \(\mu\)g/ml propidium iodide. DNA content was assessed by flow cytometry using a FACSscan (Becton Dickinson, Mountain View, CA), and the resulting data were analyzed using ModFit software (Version 1.00 for Mac, ModFit Verity Software House, Topsham, ME). Isolated nuclei were utilized in this procedure due to the tendency of PC12 cells to aggregate in solution.

**RESULTS**

The inducible p21 system used in this study was described in a previous report (29). PC12 clonal cell lines were generated containing p21\(^{WAP1}\) under control of the Lac repressor protein. When the lactose analogue IPTG was added, p21 levels increased in these cells, and this increase was followed by an irreversible cell cycle arrest. Fig. 1 demonstrates the time course of growth arrest in these cells. Shown are cell counts from one clonal line in the presence or absence of IPTG (similar results were seen in six different clonal lines). Cells grown in normal medium continued to proliferate, whereas cells grown in the presence of IPTG underwent a dramatic and complete growth arrest, shown here for up to 12 days. The aim of this study was to characterize the nature of the growth arrest induced by p21 in these cells, in particular, to compare results with changes known to occur during cell cycle arrest following NGF-induced neuronal differentiation of PC12 cells. All experiments in this study were performed in numerous clonal cell lines, with similar results obtained with each line.

To examine changes in proteins associated with cell cycle progression (cycins and Cdk proteins) following ectopic p21 induction, samples were taken prior to IPTG treatment and three days following addition of the inducer. Western blots demonstrated the increased p21 protein in these cells (Fig. 2).

Cyclins D1 and E (normally associated with G\(_{2}\)/M phase) also increased. In contrast, the mitotic cyclins (A and B1) decreased during the same time period. Similar to the mitotic cyclins, the cyclin-dependent kinases Cdc2 and Cdk4 also decreased following p21 induction (Fig. 2). Overall, the modulation of cell cycle components following p21 induction is characteristic of changes following NGF-induced differentiation (11–14).

---

**Fig. 1. Inhibition of PC12 proliferation by p21\(^{WAP1}\).** Proliferation of PC12 cells transfected with an inducible p21 construct (clone 12-64) was measured in untreated cultures (Control) or those grown in the presence of 3 mM IPTG to induce p21 expression. Relative cell number represents the ratio of cells at a given time point to the number of cells at time 0. Data represent means ± S.E. (days 1–4 (n = 5) and days 6–8 (n = 3); one experiment shown for day 12). Approximately 1.5−2.5 \(\times\) 10\(^5\) cells/35-mm culture dish were present on day 0.
To further examine growth arrest, incorporation of tritiated thymidine into DNA was measured. Previously, we have shown that there is an immediate decrease in thymidine incorporation following ectopic p21 induction (29). It was important to examine this phenomenon over a longer time course than previously described, given that PC12 cells differentiated with NGF have the capacity to incorporate thymidine into DNA, even though the cells are terminally differentiated (9, 10). An immediate decrease in thymidine incorporation was seen after incubation with IPTG to induce p21 expression (Fig. 3). In this experiment, incorporation initially decreased to near background levels; however, a subsequent increase in thymidine incorporation was seen after 3–6 days of continued growth arrest, although not to the level of actively proliferating cells (~15% of the maximum).

To interpret this late increase in thymidine incorporation into DNA, FACS analysis of DNA content was performed on proliferating and p21-arrested cells. Induction of p21 initially increased the fraction of cells with a 2n DNA content, whereas the fraction with a 3n or 4n DNA content decreased (Fig. 4A). However, the pattern began to change after 4 days of treatment, with those cells having a 2n DNA content decreasing and those having a 4n DNA content increasing. A slight increase in cells with a 3n DNA content was also observed over the same time period. In Fig. 4B, the percentage of cells with a 2n or 4n DNA content is provided for this experiment. In proliferating populations (control), the fraction of cells with a 2n DNA content was 65%. Following induction of p21 for 1 day, this increased to 81%. After 4 days, the fraction of cells with a 2n DNA content began to decrease, falling to 75% and finally to 64% after 6 days. The fraction of cells with a 4n DNA content was 12% in proliferating populations and fell to a low of 5% after 2 days. At later time points, the fraction of cells with a 4n DNA content increased, reaching 12% after 4 days and 22% after 6 days. In combination with cell counts and thymidine incorporation data, FACS analysis indicated that long-term arrest of these cells was accompanied by a resumption of DNA synthesis, leading to the generation of polyploid cells in the absence of cell division, a feature characteristic of NGF-induced differentiation of PC12 cells (9, 10).

Given these changes in DNA synthesis in the absence of proliferation, levels of p21 protein in arrested cells were examined to determine if any modulation was occurring during the time of renewed thymidine incorporation. Using an antibody directed against the amino terminus of p21 (p21(N)), protein levels were seen to increase following following IPTG induction and to remain consistently elevated for up to 8 days of treatment (Fig. 5A). Interestingly, a different pattern was detected when employing an antibody directed against the carboxyl terminus of p21 (p21(C)). In this case, p21 levels also increased following IPTG induction; however, after ~3 days, the levels began to decrease.

NGF is also known to increase p21 protein levels in PC12 cells during terminal differentiation (11–14); therefore, p21 expression during differentiation was examined to determine if a similar pattern would be detected when employing these two antibodies. As expected, NGF treatment led to a sustained increase in p21 protein when probed with antibody p21(N) (Fig. 5B). Similar to the results in the p21-inducible cell lines, probing with antibody p21(C) revealed an initial increase in p21 levels, followed by a decrease.

To attempt to understand the differences in antibody recognition of p21, lysates from cells treated with IPTG for 8 days were chosen for further study (due to the lack of p21 protein recognition by p21(C) at this time point). An obvious modification that can regulate antibody recognition is phosphorylation; therefore, lysates were treated with alkaline phosphatase to determine if phosphorylation of p21 contributes to the decreased signal observed with antibody p21(C). Western blots (Fig. 5C) revealed that recognition of p21 by this antibody was dramatically increased in lysates treated with alkaline phosphatase compared with both control lysates and lysates treated with a combination of alkaline phosphatase and the phosphatase inhibitor β-glycerophosphate. These results suggest that the carboxyl-terminal region of p21 is being phosphorylated during long-term growth arrest.

The final experiments conducted were aimed at determining the responsiveness of p21-arrested cells to mitogenic stimuli, specifically the ability of arrested cells to respond to EGF. Undifferentiated PC12 cells typically respond to EGF with an increased rate of proliferation, whereas differentiation of PC12 cells with NGF results in a population refractory to EGF treatment, i.e., no proliferative responses are observed (30). Not surprisingly, there was no increase in the number of cells
following p21 induction and treatment with EGF (data not shown). Interestingly, EGF treatment did result in neurite extension, similar to that occurring during neuronal differentiation (Fig. 6). Some short neurites were present in control, EGF-treated, and p21-arrested populations; however, numerous long neuritic processes were present following EGF treatment of p21-arrested cells in comparison with the other populations. Quantitation of the average number of neurites/cell extending at least 20 or 50 μm from the cell body showed that EGF-treated p21-arrested cells (EGF/p21) had significantly more neurites after 4 days than control, EGF-treated, or p21-arrested populations (Fig. 7). In examining neurites greater than 50 μm, EGF-treated p21-arrested cells averaged 0.6 neurites/cell body, compared with 0.003 in control, 0.07 in EGF-treated, and 0.05 in p21-arrested populations (i.e., >9-fold more neurites were present in EGF-treated cells compared with other groups). In these experiments, ~32% of EGF-treated p21-arrested cells had at least one neurite extending >50 μm, compared with <5% in all other groups (data not shown). The level of neurite outgrowth following EGF treatment of p21-
arrested cells was comparable to that seen following treatment of PC12 cells with NGF alone, although it was not as extensive as neurite development in NGF-treated p21-arrested cells (29).

**FIG. 5.** p21\(^{WAF1}\) phosphorylation during growth arrest. A, Western blot analysis of p21 expression following induction with 3 mM IPTG (clone 12-3). Cells were treated for 0–8 days, and total cellular proteins were analyzed with an amino terminus-specific (p21(N)) or carboxyl terminus-specific (p21(C)) antibody. B, Western blot analysis of p21 expression in differentiating PC12 cell cultures (parent cell line). Cells were treated with NGF for 0, 3, or 6 days, and total cellular protein was analyzed with p21(N) or p21(C). C, Western blot analysis of p21 expression in phosphatase-treated lysates. Cells treated with 3 mM IPTG for 8 days to induce p21 expression were lysed and incubated in the presence (+) or absence (−) of alkaline phosphatase (AP) and alkaline phosphatase inhibitor (API). Equal amounts of lysates were then analyzed with p21(C) or p21(N).

**FIG. 6.** Treatment of p21\(^{WAF1}\)-arrested PC12 cells with EGF leads to neurite outgrowth. Shown are photomicrographs of cells (clone 12-3) grown in normal medium (A) or in medium containing EGF (20 ng/ml) (B), 3 mM IPTG (C), or 3 mM IPTG + 20 ng/ml EGF (D). Cultures were treated with EGF for 4 days. IPTG was added to cultures for 3 days prior to addition of EGF; therefore, p21 was induced for a total of 7 days (C and D).

**FIG. 7.** Increased neuritogenesis in p21\(^{WAF1}\)-arrested, EGF-treated cells. Cells were treated as described in the legend to Fig. 6, and the number and length of neurites from cells in 8–12 random fields in three independent experiments were measured in control (CTRL), p21-arrested (p21), EGF-treated (EGF), and EGF-treated p21-arrested (EGF/p21) cultures. Shown is the average number of neurites from individual cells extending >20 or 50 μm. Data represent means ± S.E. of three independent experiments. *, \(p < 0.05\) compared with all other groups.

**DISCUSSION**

During terminal differentiation of neurons, cells must stop dividing and permanently withdraw from the cell cycle. One of the mechanisms by which this may be accomplished is increased expression of the cyclin-dependent kinase inhibitor p21\(^{WAF1}\). Treatment of PC12 cells with NGF leads to increased expression of p21 protein (11–14). As levels rise, p21 becomes associated with cyclin D1-Cdk4 and cyclin E-Cdk2 complexes, and these associations are correlated with a decrease in the kinase activities (12, 13). In neuroblastoma cell lines, NGF-induced differentiation is also accompanied by elevation of p21, and when this increase is blocked, cells fail to differentiate, leading to apoptotic cell death (31). Overall, these results suggest a role for p21 in cell cycle withdrawal accompanying terminal neuronal differentiation.

Previously, an irreversible arrest in proliferation was observed in PC12 cell lines following ectopic expression of p21 (29). In the present study, cell cycle changes following p21 induction were characterized in an attempt to determine if p21 may be responsible for numerous cell cycle changes that occur in response to NGF. The first parameters investigated were modulation of components associated with cell cycle progression. Induction of p21 leads to an increase in cyclins D1 and E and to a decrease in cyclins A and B. Decreased Cdk4 and Cdc2 expression is also observed in response to p21. Following treatment of PC12 cells with NGF, it has been consistently shown that levels of Cdc2, Cdk4, Cdc2, and cyclin B decrease (12, 13), whereas levels of cyclin D1 increase (11–14). Cyclin E levels have been shown to remain elevated (10) or to increase (11) in response to NGF, whereas cyclin A levels remain stable (10) or decrease (11, 13) (the reasons for the apparently conflicting results with cyclins A and E are unclear, although they may be due to the use of different culture conditions (13)). Similar to the proliferation arrest seen during treatment of PC12 cells with NGF, changes in cyclin proteins following p21 expression are indicative of an accumulation of cells in G1. This is not surprising given that p21 is known to block the cell cycle at this point (21); however, the down-regulation of Cdk proteins does not correlate with changes in the phase of the cell cycle, as
these proteins are typically regulated by post-translational mechanisms during normal cell cycle progression (2).

The permanent growth arrest and changes in cell cycle components induced by p21 alone are the same as effects seen in response to NGF-induced differentiation and appear to be specific to this type of growth arrest. For example, incubation of PC12 cells in a serum-free medium results in proliferation arrest (32), although no accumulation of cyclin D1 occurs (13). Treatment of PC12 cells with staurosporine (14) or EGF in conjunction with antiproliferative signals (33) can result in morphological differentiation, although again, cyclin/Cdk expression patterns are not identical to those seen following NGF treatment. Together, these results indicate that changes in cell cycle components induced by NGF were matched specifically by p21 expression and do not appear to be a result of general inhibition of cell cycle progression.

In addition to known changes in cell cycle components, a novel finding in this study is the apparent phosphorylation of p21 during growth arrest as demonstrated by Western blots. The carboxyl-terminal antibody sensitive to p21 phosphorylation is directed against amino acids 146–164, indicating that the phosphorylation most likely occurs at or near this site (within this 19-amino acid region, there are three serine residues and one threonine residue that could potentially serve as sites for phosphorylation). To confirm these results, additional experiments are required directly examining phosphorylation of p21. For example, p21 could be immunoprecipitated from 32P-labeled cells employed in this study after 1 and 8 days of induction. Not only would this be a more direct measure of phosphorylation, these studies could also be coupled with point mutations of suspected phosphorylation sites to precisely determine the amino acid residues involved in this process.

It is presently unclear what relevance this phosphorylation might have to p21 functions. Phosphorylation at this site might alter associations with proliferating cell nuclear antigen, as this region of p21 has previously been shown to be important for interaction with this protein (21), although levels of proliferating cell nuclear antigen are relatively low in p21-arrested PC12 cells (data not shown) and NGF-differentiated cells (12) compared with proliferating populations. Alternatively, phosphorylation might induce broad conformational changes, alter localization, affect protein stability, or any number of possibilities, all of which could alter function. In other studies, phosphorylation of p21 protein has been reported to occur at serines 98 and 130 (34), although no functional consequences of this modification could be determined. Finally, it should also be noted that a novel carboxyl-terminal truncation of p21 has previously been shown to be important for interaction with this protein, although levels of proliferating cell nuclear antigen are relatively low in p21-arrested PC12 cells (data not shown) and NGF-differentiated cells (12) compared with proliferating populations. Alternatively, phosphorylation might induce broad conformational changes, alter localization, affect protein stability, or any number of possibilities, all of which could alter function. In other studies, phosphorylation of p21 protein has been reported to occur at serines 98 and 130 (34), although no functional consequences of this modification could be determined. Finally, it should also be noted that a novel carboxyl-terminal truncation of p21 has been reported, and this truncation results in altered detection of p21 protein using the p21(N) and p21(C) antibodies employed in the current study (35). In the present experiments examining potential p21 phosphorylation, the percent recovery of p21 immunoreactivity following alkaline phosphatase treatment was not determined. The possibility exists that some of the differences observed between the two antibodies could be due to a truncation of the protein; indeed, phosphorylation at or near the carboxyl terminus could trigger proteolytic processing of p21. Overall, the PC12 system described in this study should be useful for determining the potential role of phosphorylation in the regulation of p21 and resulting cellular effects.

In this study, the apparent phosphorylation of p21 was temporally correlated with reinitiation of DNA synthesis. Cells initially arrested with a 2n DNA content (indicative of G1); however, a low level of tritiated thymidine incorporation was observed after long-term arrest. This was accompanied by a shift from a 2n to 4n DNA content. In previous studies, individual neurite-bearing PC12 cells have been shown to incorporate thymidine without subsequent cell division (9). Flow cytometry profiles of NGF-treated PC12 cells have revealed an initial arrest with a 2n DNA content, although over time, the fraction of cells with a 2n content decrease and those with a 4n content increase (10), similar to the results obtained in this study. Although the shift to a 4n DNA content could be indicative of an accumulation in G2 (either NGF-treated or p21-arrested populations), van Grunsven et al. (13) demonstrated that a fraction of NGF-differentiated PC12 cells were arrested in G1 with a tetraploid DNA content.

Interestingly, the generation of polyploid cells has also been observed following p21 overexpression in other systems, including megakaryocytic (36), breast carcinoma (37), and osteosarcoma (38) cell lines. In vivo overexpression of p21 in hepatocytes also resulted in the appearance of polyploid cells (39). Although the significance of polyploidy in neurons is unclear, a fraction of differentiated cerebellar Purkinje neurons become tetraploid ~12 days after becoming post-mitotic (40, 41). This study suggests the possibility that p21 may be involved in this process. Mechanistically, it has been suggested that the Rb status of the cell may contribute to the process of polyploidization in response to p21 overexpression, as Rb-negative cells undergo DNA endoreduplication, whereas Rb-positive cells do not (38). Although not sequenced, Rb protein has been considered functional in PC12 cells based on studies during differentiation (12, 42). Furthermore, Rb-negative cells showed accumulation of cyclins A and B following p21 induction, as opposed to the reductions demonstrated in this study. These results would seem to indicate that the resumption of DNA synthesis observed in PC12 cells does not result from inactivation of the Rb pathway. It would be of interest to determine if p21 phosphorylation plays a role in the endoreduplication of DNA, given the temporal correlation of this modification with reinitiation of DNA synthesis. It is possible that phosphorylation could lead to changes in associations with proliferating cell nuclear antigen, cyclin A-Cdk2, or other proteins associated with DNA synthesis. Although the levels of these proteins generally decrease following p21 expression/NGF treatment, low levels may be present across the population, or transient increases may be present in a limited number of cells, allowing for this process to occur.

The final aspect of p21-induced growth arrest examined was the ability to respond to a known PC12 mitogen, specifically EGF. This was of interest, not only to determine if cell cycle arrest could be overcome by EGF, but also because differentiated PC12 cells are known to be refractory to EGF stimulation due to a down-regulation of the EGF receptor (30). Following p21 expression, cells did not divide in the presence of EGF; however, cells did respond with neurite extension, similar to that seen in response to NGF treatment. Similar results in PC12 cells have been observed when incubated in the presence of EGF with KCl (43); cAMP, rapamycin, mimosine (33); interleukin-6 (44); staurosporine (45); or K252a (46). In the case of co-treatment with KCl, cAMP, rapamycin, and mimosine, the ability of EGF to induce differentiation responses was attributed to the antiproliferative actions of these compounds. This study supports this hypothesis, in that a direct physiological inhibition of proliferation with the cyclin-dependent kinase inhibitor p21 was sufficient to cause EGF signaling to result in neurite outgrowth. It is known that treatment of PC12 cells with EGF or NGF results in activation of similar protein-tyrosine kinase pathways, although the time course and some of the ultimate targets are not identical (47, 48). Overall, inhibition of proliferation with p21 alone was enough to cause

---

2 J. A. Erhardt and R. N. Pittman, unpublished observations.
aspects of neuronal differentiation in the presence of EGF, suggesting that tyrosine kinase pathways common between NGF and EGF are sufficient to induce differentiation if signaling pathways responsible for proliferation are inhibited. Therefore, a key difference between the effects of NGF and EGF on PC12 cells is that NGF induces growth arrest (possibly through induction of p21), allowing tyrosine kinase pathways to cause differentiation.

In this study, we have demonstrated that the cell cycle arrest induced by p21 overexpression alone is essentially identical to that induced during NGF treatment of PC12 cells. It has previously been proposed that NGF induces cell cycle arrest in PC12 cells through p21 based on its increased expression during the differentiation process (14, 49). Poluha et al. (50) demonstrated that increased p21 expression in PC12 cells following NGF treatment resulted from nitric oxide generation and that addition of nitric-oxide synthase inhibitors could block differentiation. Furthermore, overexpression of p21 in the presence of nitric-oxide synthase inhibitors could restore the differentiation response. Together, these data suggest that increased p21 expression is responsible for cell cycle arrest during neuronal differentiation of PC12 cells.

REFERENCES
1. Graña, X., and Reddy, E. P. (1995) Oncogene 11, 211–219
2. Morgan, D. O. (1995) Nature 374, 131–134
3. Pines, J. (1995) Biochem. J. 308, 697–711
4. MacLachlan, T. K., Sang, N., and Giordano, A. (1995) Crit. Rev. Eukaryotic Gene Expression 5, 127–156
5. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
6. Harper, J. W., and Elledge, S. J. (1996) Curr. Opin. Genet. Dev. 6, 56–64
7. Maier, T., and Tschicier, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
8. Pittman, R. N., Wang, S., DiBenedetto, A. J., and Mills, J. C. (1993) J. Neurosci. 13, 3669–3680
9. Ignatius, M. J., Chandler, C. R., and Shooter, E. M. (1995) J. Neurosci. 15, 434–435
10. Buchkovich, K. J., and Ziff, E. B. (1994) Mol. Biol. Cell 5, 1225–1241
11. Shinohara T., Tetsuhiro, K., Matsumine, A., Teshima, K., and Akamatsu, T. (1995) J. Biol. Chem. 270, 23031–23037
12. Yan, G., and Ziff, E. B. (1995) J. Neurosci. 15, 6200–6212
13. van Grunsven, L. A., Billon, N., Savatier, P., Thomas, A., Urdiales, J. L., and Rudkin, B. B. (1996) Oncogene 12, 1347–1356
14. Gollapudi, L., and Neet, K. E. (1997) J. Neurosci. Res. 49, 461–474
15. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
16. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 211, 90–98
17. Jiang, H., and Fisher, P. B. (1995) Mol. Cell. Differ. 1, 285–299
18. Xiong, Y., Zhang, H., and Beach, D. (1992) Cell 71, 505–514
19. Gu, Y., Tsuch, C. W., and Morgan, D. O. (1993) Nature 366, 707–710
20. Harper, J. W., Adaml, G. R., Weil, N., Keyomarsi, K., and Elledge, S. J. (1995) Cell 75, 805–816
21. Godt, A. L., Forsan, B. M., and Tynier, A. L. (1996) Proc. Soc. Exp. Biol. Med. 213, 138–149
22. Jiang, H., Lin, J., Su, Z., Collart, F. R., Huberman, R., and Fisher, P. B. (1994) Oncogene 9, 3397–3406
23. Steinman, R. A., Hoffman, B., Iro, A., Guilou, C., Liebermann, D. A., and El-Houseini, M. E. (1994) Oncogene 9, 3389–3396
24. Guo, K., Wang, J., Andrews, V., Smith, R. C., and Walsh, K. (1995) Mol. Cell. Biol. 15, 3823–3828
25. Hairey, D. O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) Science 267, 1018–1021
26. Missiro, C., Calautti, E., Ecker, R., Chin, J., Tsai, L., Livingston, D. M., and Dotti, G. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5451–5455
27. Hupper, K., Siwarski, D., Dosik, J., Cenedoli, M., Reed, S., Dock, M., Givol, D., and Muskus, J. F. (1994) Oncogene 9, 3017–3020
28. Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995) Science 267, 1024–1027
29. Erhardt, J. A., and Pittman, R. N. (1998) Oncogene 16, 443–451
30. Lazarovici, P., Oshima, M., Shavit, D., Shibutani, M., Jiang, H., Monshepiori, M., Fink, D., Movseyen, V., and Guroff, G. (1997) J. Biol. Chem. 272, 11026–11034
31. Poluha, W., Poluha, D. K., Chang, B., Brosie, N., Schonhoff, C. M., Kilpatrick, D. L., and Ross, A. H. (1996) Mol. Cell. Biol. 16, 1335–1341
32. Rudkin, B. B., Lazarovici, P., Levi, B., Abe, Y., Fujita, K., and Guroff, G. (1989) EMBO J. 8, 3319–3325
33. Mark, M. D., and Storm, D. R. (1997) J. Biol. Chem. 272, 17238–17244
34. Zhang, H., Hannon, G. J., and Beach, D. (1994) Genes Dev. 8, 1755–1758
35. Tchou, W.-H., Rom, W. N., and Tchou-Wong, K.-M. (1996) J. Biol. Chem. 271, 24156–24160
36. Kikuchi, J., Furukawa, Y., Iwase, S., Terui, Y., Nakamura, M., Kitagawa, S., Kitagawa, M., Komatsu, N., and Miura, Y. (1997) Blood 89, 3890–3899
37. Sheik, M. S., Rochefort, H., and Garcia, M. (1995) Oncogene 11, 1899–1905
38. Nicaulescu, A. R., Chen, S. C., Smeets, M., Hendst, L., Prives, C., and Reed, S. (1998) Mol. Cell. Biol. 18, 629–643
39. Wu, H., Wade, M., Krall, L., Grisham, J., Xiong, Y., and Van Dyke, T. (1996) Genes Dev. 10, 245–260
40. Lapham, L. W. (1968) Science 159, 310–312
41. Lenz, R. D., and Lapham L. W. (1970) J. Neuropathol. Exp. Neurol. 29, 43–56
42. Kilmann, D., Whittaker, K., Bishop, J. M., and Oellig, P. H. (1993) Mol. Biol. Cell 4, 353–361
43. Mark, M. D., and Storm, D. R. (1997) Neurosci. Lett. 230, 73–76
44. Wu, Y. Y., and Bradshaw, R. A. (1996) J. Biol. Chem. 271, 13033–13039
45. Raffel, S., and Bradshaw, R. A. (1995) J. Biol. Chem. 270, 7568–7572
46. Iono, P., Widmer, H. R., Hefti, F., and Kruzel, B. (1994) J. Neurochem. 63, 1235–1245
47. Bunnell, D., Radeke, M. J., and Feinstein, S. C. (1995) J. Neurosci. Res. 41, 628–639
48. Hondermarck, H., McLaughlin, C. S., Patterson, S. D., and Bradshaw, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9377–9381
49. Yan, G., and Ziff, E. B. (1997) J. Neurosci. 17, 6122–6132
50. Poluha, W., Schonhoff, C. M., Harrington, K. S., Lachyankar, M. B., Brosie, N. E., Bulseco, D. A., and Ross, A. H. (1997) J. Biol. Chem. 272, 24002–24007