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The Cyclin-Dependent Kinase Inhibitor p21^{WAF1} Is Required for Survival of Differentiating Neuroblastoma Cells

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We are employing recent advances in the understanding of the cell cycle to study the inverse relationship between proliferation and neuronal differentiation. Nerve growth factor and aphidicolin, an inhibitor of DNA polymerases, synergistically induce neuronal differentiation of SH-SY5Y neuroblastoma cells and the expression of p21^{WAF1}, an inhibitor of cyclin-dependent kinases. The differentiated cells continue to express p21^{WAF1}, even after removal of aphidicolin from the culture medium. The p21^{WAF1} protein coimmunoprecipitates with cyclin E and inhibits cyclin E-associated protein kinase activity. Each of three antisense oligonucleotides complementary to p21^{WAF1} mRNA partially blocks expression of p21^{WAF1} and promotes programmed cell death. These data indicate that p21^{WAF1} expression is required for survival of these differentiating neuroblastoma cells. Thus, the problem of neuronal differentiation can now be understood in the context of negative regulators of the cell cycle.

We are using the SH-SY5Y neuroblastoma cell line as a model for neuronal terminal differentiation (33, 48). These cells express low levels of both the low-affinity nerve growth factor (NGF) receptor and the trkA NGF receptor (2, 48). SH-SY5Y cells treated with NGF and aphidicolin, a specific and reversible inhibitor of DNA polymerases α and δ, cease proliferation and extend long neurites (25, 33). The differentiated cells require NGF for survival and, in the presence of NGF, are stable for 4 to 6 weeks. These cells express neuronal markers and cytologically resemble sympathetic neurons. In contrast, NGF alone does not stop cell proliferation and induces only slight neurite extension (8, 55). Treatment with aphidicolin does not induce neurite extension, and the cells resume proliferation following removal of aphidicolin.

Several mechanisms have been proposed for cessation of neuronal proliferation. One way by which growth arrest might occur is by down-regulating the expression of proteins required for progression of the cell cycle through the G_1-S and G_2-M transitions (24). cdc2 is the p34^{cdc2} kinase, which in conjunction with cyclin B triggers mitosis (40). The kinases cdk2 and cdk4, in association with cyclins A, D1, D2, D3, and E, are required for the G_1-S transition. Neuronal precursor cells, but not neurons in the adult brain, express cdc2, cdk2, and cyclins A and D2 (24, 31, 45, 58, 59). In contrast, cyclins D1 and E are expressed in both the developing and the adult brain (37, 58). Sympathetic neurons do not express cdc2, cdk2, or cyclin A but do express cdk4 and cyclins B, D1, D2, D3, and E (18). PC12 pheochromocytoma cells differentiated with NGF express decreased levels of cdc2 and cdk2 but continue to express cyclins A and E (6). Hence, although the details vary, down-regulation of cell cycle-associated proteins occurs in a variety of neuronal systems.

A second potential mechanism for growth arrest involves induction of p21^{WAF1}, which binds to and inhibits cdc2, cdk2, and cdk4 (16, 23, 43). Transcription of p21^{WAF1} is induced both by the tumor suppressor p53 (16, 17) and by p53-independent mechanisms (15, 26, 36, 52, 56). Induction of p53 and p21^{WAF1} by irradiation of cells with X rays blocks the cell cycle at the G_1-S transition (7, 14, 17). p21^{WAF1} is also induced in a number of hematopoetic and hepatoma cell lines undergoing differentiation (26, 56). In vivo, expression of p21^{WAF1} is enhanced as myoblasts differentiate into muscle cells (22, 47, 54). p21^{WAF1} is not present in the mitotic germinal layer of the olfactory epithelium but is expressed by olfactory neurons (47). PC12 cells differentiated with NGF express elevated levels of p21^{WAF1} (67). Senescent fibroblasts express p21^{WAF1}, and induction of an antisense RNA for p21^{WAF1} results in renewed DNA synthesis and entry of some of the senescent cells into the cell cycle (42). Furthermore, disruption of the p53-p21^{WAF1} pathway by mutation of the p53 gene is an important step in tumor carcinogenesis (64). However, unlike many other tumor types, nearly all neuroblastomas, including SH-SY5Y, carry wild-type p53 genes (10, 30, 62).

In this study, we tested the relevance of both models to neuronal differentiation of SH-SY5Y cells. We found that p21^{WAF1} is induced during differentiation but that expression of cdc2 and cdk2 is unchanged. Sustained induction of p21^{WAF1} is dependent on the presence of both NGF and aphidicolin and occurs in large part at the transcriptional level. p21^{WAF1} antisense oligonucleotides partially block p21^{WAF1} expression, leading to programmed cell death. We conclude that sustained expression of p21^{WAF1} is required for survival of differentiating SH-SY5Y cells.

MATERIALS AND METHODS

Cell culture. The neuroblastoma cell line SH-SY5Y has been described (3). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 μg of gentamicin per ml. For differentiation studies, cells were plated on Primaria (Falcon Plastics) dishes or flasks and were treated with 100 ng of NGF (2.5S; Bioproducts for Science) per ml and/or 0.3 μM aphidicolin (Sigma Chemical Co.). Fresh medium containing aphidicolin and NGF was added every 2 to 3 days.

Antisera. The following antibodies were used: anti-p21^{WAF1} monoclonal antibody 6B6 (PharMingen), anti-p53 monoclonal antibody PAb-1801 (Oncogene Science), anti-cyclin A monoclonal antibody (UBI; 05-155), anti-cyclin B rabbit serum (H. Piwnica-Worms, Beth Israel Hospital), anti-cyclin D1 rabbit serum (Y. Xiong and D. Beach, Cold Spring Harbor Laboratory), anti-cyclin E monoclonal antibody HE111 (E. Harlow, Massachusetts General Hospital), anti-cdc2 rabbit

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antiserum (H. Piwnica-Worms), anti-cdk2 rabbit antiserum (UBI; 06-148), and anti-Rb protein monoclonal antibody MAB-245 (H. Piwnica-Worms).

Western blotting (immunoblotting) and immunofluorescence microscopy. SH-SY5Y cells were mixed with sodium dodecyl sulfate (SDS)-gel electrophoresis loading buffer and boiled for 10 min. The resulting extracts (120 μg of protein per lane) were subjected to electrophoresis on a 12.5% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham) and a chemiluminescence reagent (34).

For immunofluorescence microscopy, cells were fixed at 4°C for 10 min on plastic dishes with 4% paraformaldehyde and then treated with methanol (−20°C) for 10 min. The cells were blocked at room temperature for 10 min with 0.05% Tween 20–0.1% bovine serum albumin in phosphate-buffered saline (PBS). The cells were incubated at room temperature for 45 min with 5 μg of anti-p21^{WAF1} antibody per ml in blocking buffer and washed with PBS. The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and washed with PBS. To stain the nuclei, the cells were incubated for 5 min with 10 μg of Hoechst 33342 per ml and then washed with PBS. The cells were immersed in Citifluor (Polysciences), sealed under a coverslip with fingernail polish, and examined with a Zeiss Axioplan microscope.

Northern (RNA) blotting and nuclear run-on assays. Total RNA was isolated by the single-step method of Chomczynski and Sacchi (9). Poly(A)$^+$ RNA was selected by using Poly ATtract oligo(dT) magnetic beads (Promega). The resulting RNA (4 μg per lane) was resolved by electrophoresis on a 1.0% agarose-formaldehyde gel, transferred to a Duralose membrane (Stratagene), and fixed by UV irradiation. The membranes were hybridized with [^{32}P]dCTP-random-primer-labeled probes, including pSAF1 for cdc2, pCycA for cyclin A, and pVLcycB for cyclin B, supplied by H. Piwnica-Worms of Beth Israel Hospital; pcyclinC, from S. Reed of Scripps Research Institute; pHsCycD1-H12 for cyclin D1, from Y. Xiong and D. Beach of Cold Spring Harbor Laboratory; pCITE-E for cyclin E, from A. Kuff of the Fred Hutchinson Cancer Research Center; pB5 for cdk2, supplied by L.-H. Tsai and E. Harlow of Massachusetts General Hospital; pCMVp53(WT) for p53, from K. Lam of the Massachusetts General Hospital Cancer Center; pHu145B2 for gadd45, from I. Alamo and A. Fornace of the National Cancer Institute; pHDM for HDM-2, from A. Levine of Princeton University; pBSSK-p16 for p16, from D. Beach; pZL-WAF1 for p21^{WAF1}, from W. El-Deiry and B. Vogelstein of Johns Hopkins University School of Medicine; and pET21a-hp27 for p27^{KIP1} from J. Massagué of the Memorial Sloan-Kettering Cancer Center. For quantitation, autoradiograms were analyzed with an LKB Ultroscan XL laser densitometer.

For nuclear run-on assays, nuclei were isolated with hypotonic buffer containing Nonidet P-40 (19). Nuclei were resuspended in 200 μl of glycerol storage buffer (25% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 3 mM dithiothreitol, and 50 mM Tris-HCl [pH 7.4]) at about 7.5×10^7 nuclei per ml. The run-on reaction was performed as previously described (19). RNA was extracted (9) and further purified by precipitation with trichloroacetic acid. Purified ^{32}P-labeled RNA (3.5×10^6 cpm/ml) was hybridized to linearized plasmids bearing cDNAs for p21^{WAF1} (pZL-Waf-1) (16), glyceraldehyde-3-phosphate dehydrogenase (pHcGAP) (60), or human β-actin (pHFbeta-A-1) (21) blotted onto nitrocellulose membranes. Following washing and RNase A treatment (19), radioactivity was quantitated with a Molecular Dynamics PhosphorImager.

Immunoprecipitations and kinase assays. Cells were suspended in 0.3 ml of lysis buffer (0.4% Nonidet P-40, 120 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 2 mM EDTA, 10 μg of leupeptin per ml, and 50 mM Tris-HCl [pH 8.0]). Extracts were clarified by centrifugation at 4°C for 15 min ($10{,}000 \times g$). The resulting supernatants were incubated at 4°C for 2 h with anti-cyclin E monoclonal antibody HE111. Immune complexes were collected with 30 μl of protein G-Sepharose beads (Sigma Chemical Co.) and washed twice with lysis buffer and once with a solution containing 20 mM Tris (pH 7.5) and 10 mM $MgCl_2$. Nine-tenths of the beads were mixed with SDS sample buffer containing β-mercaptoethanol and boiled. These samples were subjected to SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel) and Western blotting. The remaining one-tenth of the beads were suspended in kinase assay buffer containing histone H1 (Ambion) and [γ-^{32}P]ATP (9×10^6 cpm; Du Pont). The phosphorylation reaction mixture was incubated at 37°C for 15 min, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel).

Antisense oligonucleotides. Phosphorothioate oligodeoxynucleotides (100 μM) and Lipofectin (1 mg/ml; a 1:1 [wt/wt] mixture of *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride and dioleoyl phosphatidylethanolamine [Gibco]) were incubated at 37°C for 15 min. The oligonucleotide-Lipofectin mixture was diluted with serum-containing medium and added to the cells. In most cases, the dilution was 1:100, giving a final oligonucleotide concentration of 1 μM. Fresh oligonucleotide-containing medium was added to the cells each day. We used antisense oligonucleotides based on the p21^{WAF1} coding sequence, including AS-IC, which is complementary to the region around the initiation codon (5′-TCC CCA GCC GGT TCT GAC AT-3′); AS-MID, from the middle of the coding region (5′-CCT CCA GTG GTG TCT CGG TG-3′); and AS-3′, from the 3′ end (5′-TGT CAT GCT GGT CTG CCG CC-3′). These oligonucleotides were purchased from Oligos, Etc. As controls, we used an 18-mer oligonucleotide (control 1; 5′-TGG ATC CGA CAT GTC AGA-3′) and a 21-mer antisense oligonucleotide directed against *Mycobacterium tuberculosis* (5′-CGC TTC ATC CTG CCG TGT CGG-3′). The anti-*M. tuberculosis* oligonucleotide was synthesized at the Worcester Foundation by V. Metelev and P. Zamecnik, and in this paper, we refer to this oligonucleotide as control 2.

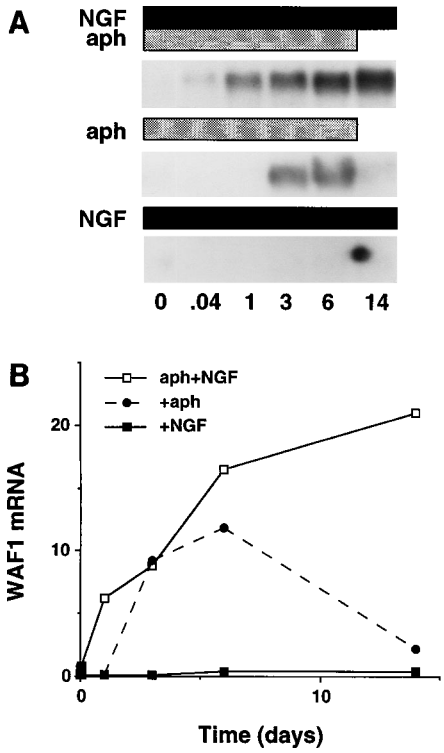

FIG. 1. Effect of NGF and aphidicolin (aph) on expression of p21^{WAF1}. (A) SH-SY5Y cells were treated for various periods with aphidicolin-NGF, aphidicolin alone, or NGF alone and used to prepare poly(A)$^+$ RNA for Northern blotting (4 μg of RNA per lane). Numbers below gels indicate days of treatment. (B) The autoradiograms were scanned, and quantitative results are displayed. aph+NGF, control cells (0) or cells treated with aphidicolin-NGF for 1 h (0.04 day), 1 day, 3 days, or 6 days. For the 14-day sample, cells were fully differentiated by treatment with aphidicolin-NGF for 6 days and then with NGF for 8 days. +aph, 0 to 6 days of treatment with aphidicolin. For the 14-day sample, cells were treated with aphidicolin for 6 days and then with normal growth medium for 8 days. +NGF, 0 to 6 days of treatment with NGF. For the 14-day sample, cells were treated with NGF for 14 days.

RESULTS

Induction of p21^{WAF1}. To examine the role of p21^{WAF1} in neuroblastoma cell differentiation, expression of the mRNA for p21^{WAF1} was assessed by Northern blotting (Fig. 1). Expression of p21^{WAF1} was up-regulated following treatment for 1 h with aphidicolin-NGF and further increased as the aphidicolin-NGF treatment progressed. On day 6, the cell medium was changed from aphidicolin-NGF medium to medium containing only NGF. Despite the removal of aphidicolin from the medium, expression of p21^{WAF1} was slightly greater on day 14 than on day 6. Treatment with aphidicolin alone also induced p21^{WAF1} expression, but following removal of aphidicolin from the medium on day 6, expression greatly declined. Treatment with NGF alone did not induce expression of p21^{WAF1}. Ethidium bromide staining of the RNA gel prior to Northern transfer, as well as rehybridization of filters with a β-actin probe, confirmed that the RNA was intact (data not shown). These results demonstrate that sustained expression of p21^{WAF1} mRNA is specifically associated with terminal differentiation of SH-SY5Y cells.

Because the p53 tumor suppressor is known to induce tran-

TABLE 1. Expression of cell cycle-associated gene products by SH-SY5Y cells[a]

Cell cycle protein	Production in:			
	Control cells		Aphidicolin-NGF-treated cells	
	mRNA	Protein	mRNA	Protein
p53	+	+	+	+++
gadd45	+	ND	+	ND
hdm-2	+	ND	+	ND
$p16^{INK4}$	−	ND	−	ND
$p21^{WAF1}$	+	+	+++	+++
$p27^{KIP1}$	+	ND	+++	ND
Cyclin A	+++	++	+	+
Cyclin B	+++	++	+++	++
Cyclin C	++	ND	+	ND
Cyclin D1	++	++	++	++
Cyclin E	++	ND	++	ND
cdc2	+++	+++	+++	+++
cdk2	++	++	++	++
Rb	ND	++	ND	++

[a] Data are from Northern and Western blot analyses. These experiments were qualitatively scored as follows: −, negative; +, only weakly detected; ++, easily detected; +++, intense signal. ND, not done. The aphidicolin-NGF-treated cells were incubated with aphidicolin (0.3 μM) and NGF (100 ng/ml) for 6 days and then with NGF alone for 6 days.

scription of $p21^{WAF1}$ (16, 17), we measured expression of the mRNA for p53. Treatment of the neuroblastoma cells with aphidicolin-NGF did not induce expression of the mRNA for p53 (Table 1).

We next determined whether levels of the p53 and $p21^{WAF1}$ proteins were elevated. Treatment of SH-SY5Y cells with aphidicolin-NGF enhanced the levels of both p53 (10-fold) and $p21^{WAF1}$ (20-fold) (Fig. 2). Expression of p53 and $p21^{WAF1}$ proteins persisted following removal of aphidicolin from the medium but at slightly lower levels. Treatment of cells with aphidicolin alone up-regulated p53 (7-fold) and $p21^{WAF1}$ (14-fold) levels (Fig. 2). Following removal of aphidicolin from the medium, expression returned to original levels. Treatment of cells with NGF alone did not induce expression of p53 or $p21^{WAF1}$.

We assessed whether the $p21^{WAF1}$ expressed in SH-SY5Y cells interacts with cyclin-dependent kinases. Anti-cyclin E antibody immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. $p21^{WAF1}$ was detected in immunoprecipitates from cells treated with aphidicolin-NGF for 6 days and from cells harvested on day 14, 8 days following removal of aphidicolin from the medium (Fig. 3). $p21^{WAF1}$ was detected in anti-cyclin E immunoprecipitates from cells treated with aphidicolin alone (6 days), but the levels of cyclin E-associated $p21^{WAF1}$ declined following removal of aphidicolin. In the case of cells treated with NGF alone, $p21^{WAF1}$ did not coimmunoprecipitate with cyclin E.

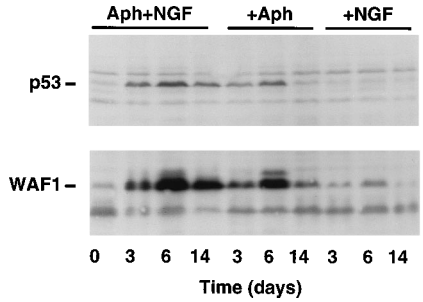

FIG. 2. Treatment with aphidicolin (aph)-NGF enhances expression of p53 and $p21^{WAF1}$ proteins. The details of the treatment of the SH-SY5Y cells are described in the legend to Fig. 1. For Western blotting, the filter was cut in half and incubated with anti-p53 antibody or anti-$p21^{WAF1}$ antibody. If the primary antibodies were omitted, then the p53 and $p21^{WAF1}$ bands were not detected (data not shown).

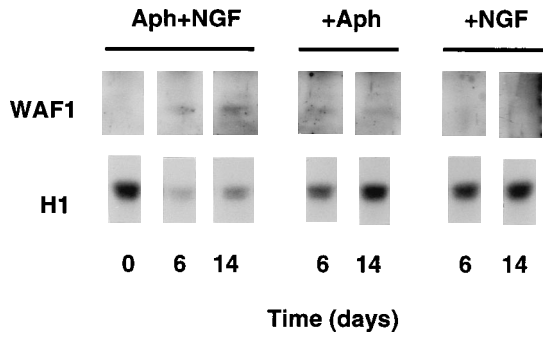

FIG. 3. Association of $p21^{WAF1}$ with cyclin E-containing complexes results in diminished protein kinase activity. The details of the treatment of the SH-SY5Y cells are described in the legend to Fig. 1. Immunoprecipitates prepared with anti-cyclin E antibody were used for Western blotting with an anti-$p21^{WAF1}$ antibody or for a protein kinase assay with histone H1 as the substrate. aph, aphidicolin.

When histone H1 was used as the substrate, the anti-cyclin E immunoprecipitates were assayed for protein kinase activity (Fig. 3). For cells treated with aphidicolin-NGF for 6 days, protein kinase activity was decreased relative to that of control cells. On day 14, 8 days after removal of aphidicolin from the medium, protein kinase activity remained decreased. Treatment of cells with aphidicolin alone decreased protein kinase activity, but to a lesser degree than treatment with aphidicolin-NGF, and following removal of aphidicolin, protein kinase activity returned to original levels (Fig. 3). Treatment of cells with NGF alone had no effect on protein kinase activity associated with cyclin E immunoprecipitates (Fig. 3). The inverse correlation between $p21^{WAF1}$ expression and kinase activity suggests that $p21^{WAF1}$ plays a major role in regulating cyclin-dependent kinases during differentiation.

We then determined whether the increases in $p21^{WAF1}$ expression resulted from transcriptional and/or posttranscriptional events. Recent studies have implicated both p53-dependent (16, 17) and p53-independent (15, 26, 36, 52, 56) regulation of $p21^{WAF1}$ gene expression. To examine this question, transcription of $p21^{WAF1}$ was measured by the nuclear run-on assay (Fig. 4). Transcription of $p21^{WAF1}$ increased four- to eightfold after 3 days of treatment with aphidicolin-NGF. The magnitude of the increased transcription was similar to

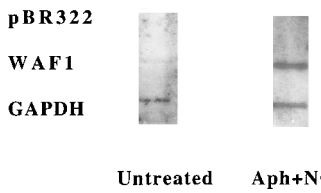

FIG. 4. Nuclear run-on assays. Cells were treated with aphidicolin (aph)-NGF for 3 days prior to preparation of nuclei. Washed membranes were exposed to X-ray film for 3 days and subsequently quantitated by phosphorimaging. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

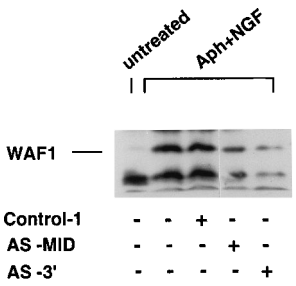

FIG. 5. Antisense oligonucleotides targeted to p21^{WAF1} mRNA inhibit expression of p21^{WAF1}. Untreated cells or cells treated for 6 days with aphidicolin (aph)-NGF with no oligonucleotide, with control 1 oligonucleotide, or with one of the p21^{WAF1} antisense oligonucleotides were extracted, and the extracts were analyzed by Western blotting.

that for induction of p21^{WAF1} mRNA and suggests that increased transcription accounts in large part for the regulation of p21^{WAF1} during differentiation of SH-SY5Y cells.

p21^{WAF1} is required for survival of differentiating neuroblastoma cells. To test the role of p21^{WAF1} in neuroblastoma cell differentiation, we used three different antisense phosphorothioate oligodeoxynucleotides to block expression of p21^{WAF1}. In initial experiments, we found that antisense oligonucleotides had no effect on p21^{WAF1} expression (not shown). Hence, we delivered the antisense oligonucleotides via the cationic detergent Lipofectin, which enhances entry of oligonucleotides into cells (49, 65). Lipofectin alone had no effect on cell viability or neuronal differentiation (data not shown). As judged by Western blotting, expression of p21^{WAF1} induced by treatment with aphidicolin-NGF was reduced two- to threefold by antisense oligonucleotides AS-MID and AS-3′ but not by a control oligonucleotide (Fig. 5). In other experiments, treatment with antisense oligonucleotide AS-IC reduced p21^{WAF1} expression (2.0- to 2.5-fold) (not shown). The effect of antisense oligonucleotide AS-IC on p21^{WAF1} expression was independently assessed by immunofluorescence microscopy. AS-IC, but not a control oligonucleotide, decreased p21^{WAF1} immunostaining that was localized in nuclei (data not shown), consistent with earlier studies on subcellular localization of p21^{WAF1} (17, 22).

The antisense oligonucleotides had no apparent effect on the morphology (data not shown) or proliferation (Fig. 6A) of control neuroblastoma cells. In contrast, the number of cells surviving treatment with antisense oligonucleotide-aphidicolin-NGF was much lower than the number surviving treatment with aphidicolin-NGF (Fig. 6B). Cell death began on the fourth day of treatment with antisense oligonucleotide-aphidicolin-NGF, roughly coinciding with commitment of these cells to differentiation (48). Of those cells which did survive, nearly all had neuronal morphologies (data not shown). Cells were stained with Hoechst 33342 to assay for apoptotic bodies, which are characteristic of programmed cell death (20). The percentage of cells treated with antisense oligonucleotide-aphidicolin-NGF with apoptotic bodies was approximately twice that of cells treated with aphidicolin-NGF or control 2 oligonucleotide-aphidicolin-NGF (Fig. 7). Hence, the decreased survival of cells treated with antisense oligonucleotide-aphidicolin-NGF is coincident with an increased rate of programmed cell death.

Roles of other cell cycle-associated proteins. Expression of cell cycle-associated gene products other than p21^{WAF1} was assessed (Table 1 and Fig. 8). Expression of p27^{KIP1}, another inhibitor of cyclin-dependent kinases, increased during differentiation. Expression of gadd45, hdm-2, and cyclin D1 did not change, even though p53 induces expression of their mRNAs in other cell types (32). hdm-2 is the human homolog of mdm-2, a murine p53-associated protein (39). Expression of cyclin A was down-regulated in neuroblastoma cells differentiated with aphidicolin-NGF. This change, occurring after 6 days of treatment (Fig. 8), was slower than that for p21^{WAF1} (Fig. 1) as well as changes previously reported for trkA and low-affinity NGF receptors (48). There also was a slight down-regulation of the mRNA for cyclin C (Fig. 8), occurring with a time course similar to that for cyclin A. Figure 9 summarizes the changes in gene expression during differentiation of SH-SY5Y.

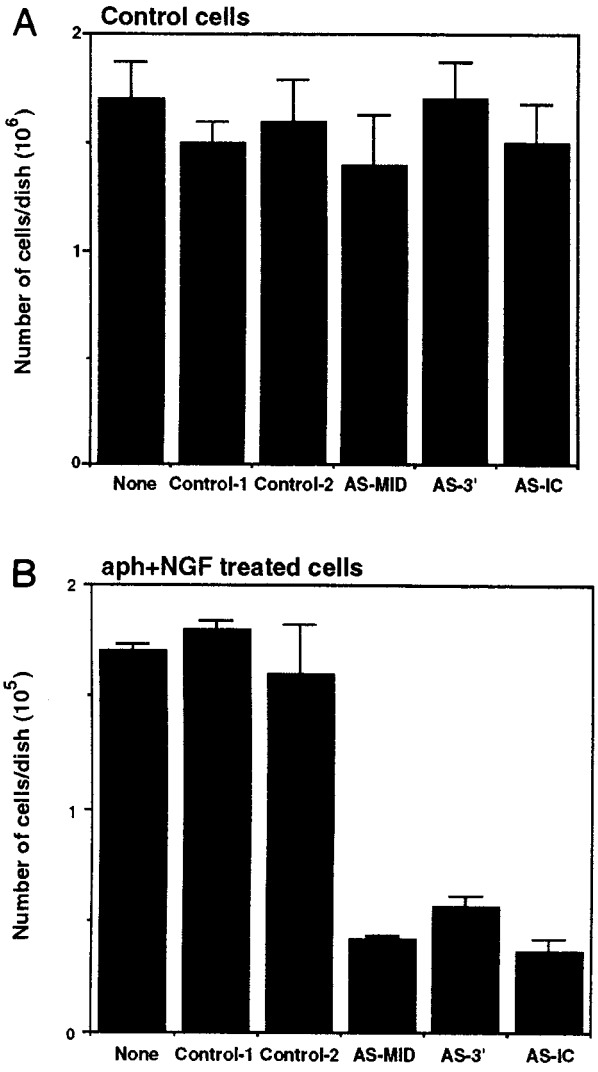

FIG. 6. Antisense oligonucleotides for p21^{WAF1} enhance cell death in SH-SY5Y cells treated with aphidicolin (aph)-NGF but not in control cells. (A) Control cells (1.5×10^5 per dish) were treated for 12 days with one of the control oligonucleotides or one of the anti-p21^{WAF1} antisense oligonucleotides, and the cell number per dish was then determined with a hemocytometer. (B) Aphidicolin-NGF with or without oligonucleotides was added to cells. After 12 days, the cells were counted. Note that the scales for panels A and B are different. There are fewer aphidicolin-NGF-treated cells because they did not proliferate.

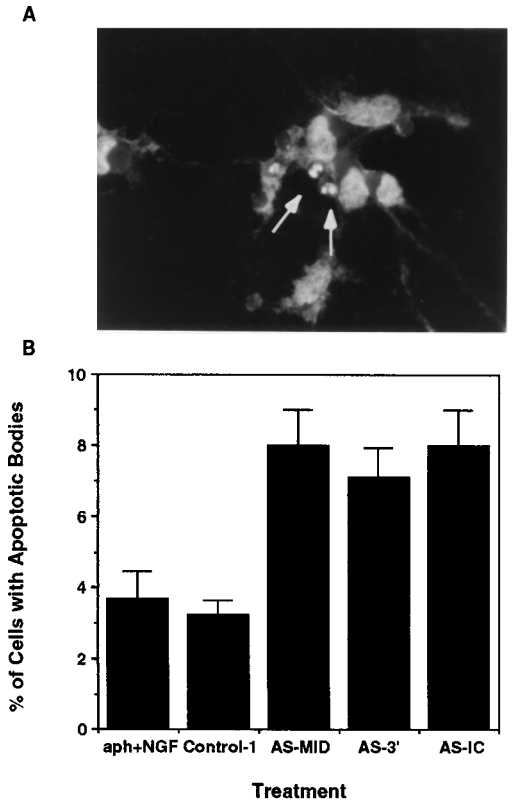

FIG. 7. Antisense oligonucleotides for p21^{WAF1} in conjunction with aphidicolin (aph)-NGF treatment increase the number of apoptotic bodies. Cells were treated for 6 days with aphidicolin-NGF in the absence of oligonucleotides (aph+NGF), in the presence of control 1 oligonucleotide, or in the presence of one of the anti-p21^{WAF1} antisense oligonucleotides. The cells then were stained with Hoechst 33342 (10 μg/ml). (A) Examples of apoptotic bodies (arrows) in cells treated with antisense oligonucleotide AS-IC–aphidicolin–NGF. (B) Quantitation of apoptotic bodies (values are means with standard deviations).

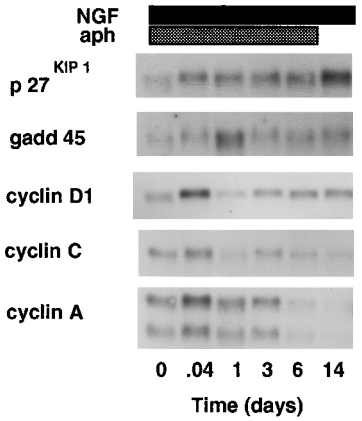

FIG. 8. Treatment of SH-SY5Y cells with aphidicolin (aph)-NGF results in up-regulation of p27^{KIP1}, no change in expression of gadd45 and cyclin D1, a modest down-regulation of cyclin C, and a substantial down-regulation of cyclin A. Cells were treated through day 6 with aphidicolin-NGF and then for days 7 to 14 with NGF. Northern blots were prepared with poly(A)$^+$ RNA (4 μg per lane). The apparent increase in expression of cyclin D1 after 1 h of treatment is due to uneven loading of the gel and did not reproduce in other experiments.

DISCUSSION

The principal results of this study are that aphidicolin and NGF act synergistically to induce expression of p21^{WAF1} and that, in turn, p21^{WAF1} is required for survival of differentiating SH-SY5Y cells. Only a small fraction of cells treated with antisense oligonucleotides specific for p21^{WAF1} differentiate. The remainder of the antisense-oligonucleotide-treated cells undergo programmed cell death.

Regulation of p21^{WAF1} expression. Up-regulation of p53 protein is probably the first step in aphidicolin-NGF-induced differentiation of neuroblastoma cells. There is no change in levels of mRNA for p53, suggesting that aphidicolin-NGF treatment results in more efficient translation of p53 mRNA or enhanced stability of the p53 protein. These results are consistent with an earlier report that treatment of lymphoblastoid cells with aphidicolin increases levels of p53 protein (27). Since the elevated expression of p21^{WAF1} observed here appears to occur at the transcriptional level, it is likely that p53 up-regulates expression of p21^{WAF1} by binding to the two p53-binding sites in the p21^{WAF1} promoter (16).

An intriguing question raised by these studies is why these neuroblastoma cells continue to express p53 and p21^{WAF1} following transfer from aphidicolin-NGF medium to medium with NGF alone. One possibility is that treatment with aphidicolin-NGF results in enhanced NGF-induced signalling which continues after removal of aphidicolin. Rudkin et al. (50) report that responses to NGF increase for PC12 cells synchronized in the G_1 phase of the cell cycle. Aphidicolin blocks the cell cycle at G_1-S and, thereby, might enhance the response of SH-SY5Y cells to NGF. In addition, treatment of SH-SY5Y cells with aphidicolin-NGF up-regulates the mRNAs for the low-affinity NGF receptor and the trkA NGF receptor, which may enhance NGF-triggered signal transduction (48). A similar mechanism is proposed for developing neurons, in which aphidicolin enhances differentiation of sympathetic neuroblasts by inducing expression of trkA (61).

p21^{WAF1} and cell survival. Treatment of differentiating neuroblastoma cells with p21^{WAF1} antisense oligonucleotide-Lipofectin decreases both expression of p21^{WAF1} protein and cell survival. Antisense treatment of control cells which express

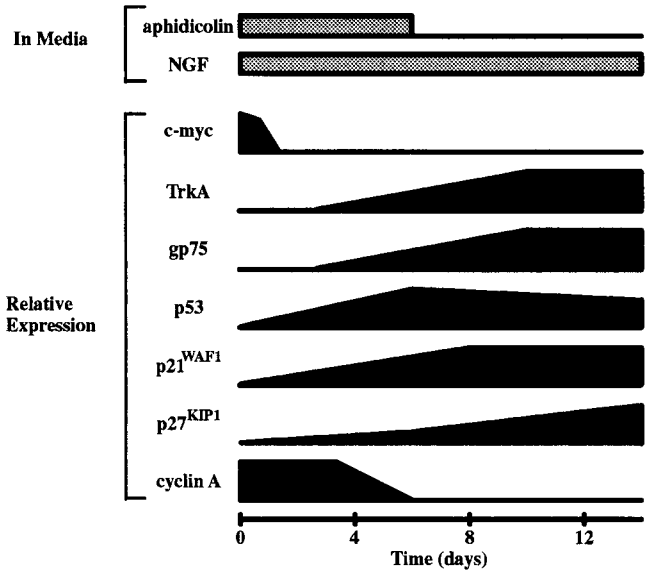

FIG. 9. Summary of changes in expression during differentiation of SH-SY5Y cells. Results are mRNA levels determined by Northern blotting, except those for p53, which are protein levels determined by Western blotting. There was a slight down-regulation of cyclin C that is not shown.

very low levels of $p21^{WAF1}$ has no apparent effect on viability or proliferation. Hence, $p21^{WAF1}$ is not required for neuroblastoma cell proliferation but is required for survival of differentiating SH-SY5Y cells.

The mechanism of death for cells treated with antisense oligonucleotide-aphidicolin-NGF is suggested by the elevated numbers of apoptotic bodies, i.e., cells with fragmented nuclei and/or condensed chromatin. Apoptotic bodies are characteristic of programmed cell death (20). However, another attribute of programmed cell death, internucleosomal fragmentation of DNA, was not detected by the DNA ladder method (data not shown) (4). Recently, programmed cell death in the absence of internucleosomal degradation has been observed for several other systems (51), and, instead, DNA may split into larger fragments (30 to 300 kb) corresponding to chromatin loop domains (44, 46). Hence, despite the apparent lack of internucleosomal degradation, it is very likely that the $p21^{WAF1}$ antisense oligonucleotide induces programmed cell death.

$p21^{WAF1}$ may enhance survival of differentiating cells by a relatively simple mechanism. Activation of cyclin-dependent kinases plays a role in the induction of some, but possibly not all, forms of apoptosis (35, 53). Hence, we propose that $p21^{WAF1}$, by inhibiting cyclin-dependent kinases, directly blocks initiation of programmed cell death. Partial inhibition of $p21^{WAF1}$ expression by the antisense oligonucleotide may be sufficient to decrease survival, since it is known that a molar excess of $p21^{WAF1}$ is required to inhibit cyclin-dependent kinases (68).

We have emphasized herein the role of $p21^{WAF1}$ in differentiation of neuroblastoma cells, but other cell cycle-associated proteins also may play a role. We noted a down-regulation of cyclin A and an up-regulation of $p27^{KIP1}$, another cyclin-dependent kinase inhibitor. In future studies, we will test the roles of cyclin A and $p27^{KIP1}$ in aphidicolin-NGF-induced differentiation of neuroblastoma cells.

Neuroblastomas and neural development. The role of $p21^{WAF1}$ in neuronal differentiation may be relevant to the clinical course of neuroblastoma progression. Neuroblastomas have the highest rate of spontaneous regression of any human tumor, probably because of differentiation of tumor cells into neuron-like cells (28). The best prognosticator for this disease is expression of the trkA NGF receptor (13, 29, 41, 57). Patients whose tumors express significant levels of trkA have a good chance for survival. Patients whose tumors lack trkA respond poorly to therapy. In addition, nearly all neuroblastomas bear wild-type, functional p53 genes (10, 30, 62). p53 protein expressed by differentiated neuroblastomas is localized in the nucleus (38); however, p53 protein in undifferentiated neuroblastomas is sequestered in the cytoplasm and, therefore, is probably inactive. Hence, at least some of the gene products required for differentiation of cultured SH-SY5Y cells are also expressed by neuroblastomas in vivo and are associated with favorable clinical outcomes. Further studies are required to determine whether expression of $p21^{WAF1}$ in human neuroblastoma tumors is also associated with a favorable prognosis.

Although the evidence is incomplete, it is likely that p53 and $p21^{WAF1}$ also play important roles in neural development. One might question this assertion, since mice lacking either p53 or $p21^{WAF1}$ are viable (5, 11, 12). However, nature tends to use multiple pathways for important processes, a redundancy that may confuse simple interpretations of gene knockout experiments (66). For example, there are multiple proteins that induce muscle differentiation, and mice lacking only one of these genes show normal phenotypes. However, mice lacking two of these genes develop little or no muscle. In fact, there is evidence for a role of p53 in neural development. $p53^{-/-}$ mice have an unusually large number of birth defects, including failure of neural tube closure (1). In addition, primary cultures of $p53^{-/-}$ peripheral neurons show decreased dependence on neurotrophins for survival (63). Future studies will likely demonstrate additional roles for p53 and $p21^{WAF1}$ in neural development, probably involving regulation of cell proliferation and death.

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