

# Cyclin-dependent Kinases Participate in Death of Neurons Evoked by DNA-damaging Agents

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**Abstract.** Previous reports have indicated that DNA-damaging treatments including certain anticancer therapeutics cause death of postmitotic nerve cells both in vitro and in vivo. Accordingly, it has become important to understand the signaling events that control this process. We recently hypothesized that certain cell cycle molecules may play an important role in neuronal death signaling evoked by DNA damage. Consequently, we examined whether cyclin-dependent kinase inhibitors (CKIs) and dominant-negative (DN) cyclin-dependent kinases (CDK) protect sympathetic and cortical neurons against DNA-damaging conditions. We show that Sindbis virus-induced expression of CKIs p16<sup>ink4</sup>, p21<sup>waf1/cip1</sup>, and p27<sup>kip1</sup>, as well as DN-Cdk4 and 6, but not DN-Cdk2 or 3, protect sympathetic neurons

against UV irradiation- and AraC-induced death. We also demonstrate that the CKIs p16 and p27 as well as DN-Cdk4 and 6 but not DN-Cdk2 or 3 protect cortical neurons from the DNA damaging agent camptothecin. Finally, in consonance with our hypothesis and these results, cyclin D1-associated kinase activity is rapidly and highly elevated in cortical neurons upon camptothecin treatment. These results suggest that postmitotic neurons may utilize Cdk4 and 6, signals that normally control proliferation, to mediate death signaling resulting from DNA-damaging conditions.

Key words: apoptosis • CDK • neuronal • cell cycle • DNA damage

NUMEROUS anticancer therapeutics activate death processes by creating DNA damage in a manner that is frequently dependent on the cell's proliferative capacity (Gorczyca et al., 1993). Recently, however, several reports have indicated that DNA-damaging agents also activate death programs in terminally differentiated postmitotic neurons (Winkelman and Hines, 1983; Wallace and Johnson, 1989; Martin et al., 1990; Morris and Geller, 1996; Park et al., 1997a, 1998; Gill and Windebank, 1998). Examples include irradiation (Enokido et al., 1996), the S phase inhibitor cytosine arabinoside (AraC)<sup>1</sup> (Winkelman and Hines, 1983; Wallace and Johnson, 1989; Martin et al., 1990; Tomkins et al., 1994; Park et al., 1998), the

DNA topoisomerase-II inhibitors etoposide, teniposide, and mitoxantrone (Nakajima et al., 1994; Tomkins et al., 1994), cisplatin (Gill and Windebank, 1998), and camptothecin, a topoisomerase-I inhibitor (Morris and Geller, 1996; Park et al., 1997a). Interestingly, several of these agents cause peripheral neuropathies and neurodegeneration (Winkelman and Hines, 1983; Baker et al., 1991; Vogel and Horoupian, 1993; Mansfield and Castillo, 1994). Accordingly, it is important to understand how these agents cause neuronal death. Recent evidence suggests that cell death by these agents is subsequent to formation of DNA strand breaks (Morris and Geller 1996; Morris, E.J., D.S. Park, J.C. Dreixler, L.A. Greene, and H.M. Greene. 1997. *Soc. Neurosci. Abstr.* 23:881). However, the downstream events that occur after treatment with such genotoxic agents remain unclear.

Cell cycle components may play a role in apoptotic signaling of proliferating cells induced to die by DNA damage. For example, increased cdc2 (Shimizu et al., 1995) and cyclin E-associated (Ping Dou et al., 1995) kinase activities in response to DNA-damaging agents have been demonstrated. Other evidence suggests that cell cycle components also play a role in certain cases of apoptotic

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1. *Abbreviations used in this paper:* AraC, cytosine arabinoside; CAT, chloramphenicol acetyl transferase; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; CNS, central nervous system; DN, dominant-negative; pRB, retinoblastoma protein.

death of postmitotic neurons. For instance, neuronal apoptosis caused by loss of trophic support is accompanied by changes in cyclin-dependent kinase (CDK) activity and cyclin expression (Brooks, S.F., L.A. Gibson, and L.L. Rubin. 1993. *Soc. Neurosci. Abstr.* 19:885; Freeman et al., 1994; Gao and Zalenka, 1995) and agents that inhibit cell cycle progression, including G1/S blockers (but not S and M phase blockers) (Farinelli and Greene, 1996), as well as the CDK inhibitors, flavopiridol and olomoucine (Park et al., 1996a), promote survival of neuronal PC12 cells and sympathetic neurons deprived of serum and/or NGF. Moreover, expression of the CDK inhibitors p16, p21, and p27 as well as of dominant-negative (DN) forms of CDK4 and 6 inhibits death of sympathetic neurons caused by NGF withdrawal (Park et al., 1997b).

Because cell cycle components appear to play a role in death of proliferating cells induced by DNA damage as well as in death of NGF-deprived postmitotic neurons, we hypothesized that neuronal death resulting from DNA-damaging agents may also use components that normally control cell cycle progression in proliferating cells (Park et al., 1997a, 1998). In support of this model, we have previously shown that pharmacological G1/S blockers and CDK inhibitors inhibit death of neuronal PC12 cells and sympathetic neurons exposed to DNA-damaging agents including UV irradiation, camptothecin, and AraC (Park et al., 1997a, 1998).

The present study had two major aims. The first was to confirm the potential role that our pharmacologically based studies have suggested for cell cycle-associated molecules, and in particular, cyclin-dependent kinases, in neuronal apoptosis evoked by DNA damage. The second was to identify at least several of the molecules in question. To achieve these ends, we used the Sindbis virus to target specific cell cycle regulatory genes to cultured rat sympathetic and cortical neurons. Sindbis virus, a positive-sense RNA alphavirus is neuronotropic and replication competent and, by use of a double subgenomic Sindbis promoter, permits expression of heterologous genes (Xiong et al., 1989; Levine et al., 1991, 1993, 1996; Hahn et al., 1992; Piper et al., 1992; Schlesinger, 1993; Cheng et al., 1996; Joe et al., 1996). It was previously shown that Sindbis virus efficiently targets heterologous genes to cultured sympathetic neurons and is an effective tool to study the role of cell cycle genes in death of sympathetic neurons induced by NGF deprivation (Park et al., 1997b).

Because previous pharmacologic evidence suggested that CDK activity is required for neuronal death resulting from DNA damage (Park et al., 1997a, 1998), we focused our studies in this direction. The CDK family, which includes Cdk2, Cdk3, Cdk4/6, and Cdc2 among others, is an important group of molecules that regulate cell proliferation (Pines, 1993). In addition, two classes of mammalian cyclin-dependent kinase inhibitors (CKIs) have been described (for review see Sherr and Roberts, 1995). Cip/kip members, which include p21, p27, and p57, inhibit the activities of a wide range of CDK-cyclin complexes. In comparison, ink4 family members, including p15, p16, p18, and p19, appear to primarily inhibit the activity of Cdk4/6-cyclin D complexes. In the present studies we examined the role of CDKs in death of neurons caused by DNA damaging agents. To do so, we used Sindbis virus-mediated

expression of CKIs as well as of DN forms of individual G1 CDKs and evaluated cyclin D1-associated kinase activity.

## Materials and Methods

### Generation of Recombinant Sindbis

The coding regions of p16 (Serrano et al., 1993), p21 (Harper et al., 1993), and p27 (Polyak et al., 1994) were subcloned into the BstEII site of the DSTEQ12 Sindbis virus vector (Joe et al., 1996) downstream of a double subgenomic Sindbis viral promoter. The coding regions of DN cdk2, 3, 4, and 6 (van den Heuvel and Harlow, 1993) and the single chain ScFv control (R6) cDNA was inserted into the XbaI site of the DSTEQ12 vector. The putative DN forms of the CDKs have been previously reported as inactivating Asp to Asn point mutations in the kinase domain (van den Heuvel and Harlow, 1993). The CAT recombinant viruses were generated previously (Cheng et al., 1996; Levine et al., 1996). FLAG tags (ATGGACTACAAGGACGATGATGACAAA) were introduced at the 3' end of the coding region of p27, p16, DN Cdk2, DN Cdk3, DN Cdk4, and DN Cdk6. Control non-expressing vectors of the CDK inhibitors were generated by eliminating the initiating codon of each inhibitor and in the case of p16, p21, Cdk3, Cdk4, and Cdk6, introducing a premature stop codon shortly after the second methionine in each coding region (Park et al., 1997b). All mutations, deletions, and FLAG tags were introduced by PCR and confirmed by sequencing. Viral particles were generated by *in vitro* translation and transfection into BHK cells and titered by plaque assay as previously described (Joe et al., 1996).

### Culture and Survival Assay of Rat Sympathetic Neurons

Primary cultures of rat sympathetic neurons were obtained from dissociated superior cervical ganglia of postnatal day 1 rats (strain; Harlan Sprague Dawley Inc., Indianapolis, IN) as described previously (Park et al., 1996b). The cells were plated in 0.5 ml of medium per well in collagen-coated 24-well dishes at a density of  $\sim 0.5$  ganglia/well ( $\sim 10,000$  neurons/well). The growth medium was RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS) and 60 ng/ml mouse NGF (Sigma Chemical Co., St. Louis, MO). To eliminate non-neuronal cells, a mixture of uridine and 5-fluorodeoxyuridine (10  $\mu$ M each) were added to the cultures on the following day. 3 d after plating, the neurons were infected with Sindbis virus (plaque-forming units per cell of 1 to 2) in 0.2 ml of RPMI 1640 media containing 2% heat-inactivated horse serum. After 1 h of infection, 0.3 ml of RPMI 1640 medium containing 16% heat-inactivated horse serum was added. The cultures were then treated immediately with 100  $\mu$ M AraC or left to incubate overnight before UV irradiation (300 J/m<sup>2</sup>). To achieve the latter, each well containing 200  $\mu$ l of medium containing NGF was exposed in a Stratolinker (Stratagene, La Jolla, CA). After irradiation, 300  $\mu$ l of additional medium containing NGF with or without drug was added to each well. At appropriate times, the numbers of viable, phase bright neurons were determined by strip counting as previously described (Rydel and Greene, 1988).

### Culture and Survival of Cortical Neurons

Rat cortical neurons were cultured from embryonic day 18 rats as previously described (Friedman et al., 1993). The neurons were plated into 24-well dishes ( $\sim 200,000$  cells/well) coated with polylysine in serum-free medium (N2/MEM [1:1] supplemented with 6 mg/ml D-glucose, 100  $\mu$ g/ml transferrin, 25  $\mu$ g/ml insulin, 20 nM progesterone, 60  $\mu$ M putrescine, 30 nM selenium). 1 d after plating, the neurons were infected with virus at a multiplicity of infection of  $\sim 0.5$  and incubated overnight. The medium was then exchanged with serum-free medium supplemented with 10  $\mu$ M camptothecin where appropriate. At appropriate times of culture under the conditions described in the text, cells were lysed and the numbers of viable cells were evaluated as previously described (Rukenstein et al., 1991). All experimental points are expressed as a percentage of cells plated on day 0 and are reported as mean  $\pm$  SEM ( $n = 3$ ).

### Immunofluorescence

Sympathetic neurons or cortical neurons were dissociated and cultured, as described above, in 6-well plates at a density of 2 ganglion/well (sympa-

thetic neurons) or 200,000 cells/well (cortical neurons). After various times of infection, neurons were fixed with 100% ethanol for 20 min at  $-20^{\circ}\text{C}$ , blocked with PBS containing 2% horse serum, and incubated with anti-FLAG primary antibody (cat No. IBI3010 [1:20 dilution]; Fisher Scientific Co., Pittsburgh, PA) and FITC-conjugated horse anti-mouse secondary antibody (1:50 dilution; Vector Labs, Inc., Burlingame, CA).

### Western Blot Analyses

Cortical neurons were dissociated and cultured as described above. 24 h after infection, the neurons were harvested in Laemmli buffer and 50  $\mu\text{g}$  of protein were loaded onto SDS-polyacrylamide gels, and then transferred onto nitrocellulose membrane as previously described (Cunningham et al., 1997). Blots were probed with anti-FLAG antibody (10  $\mu\text{g}/\text{ml}$ ).

### Cyclin D1-associated Kinase Assay

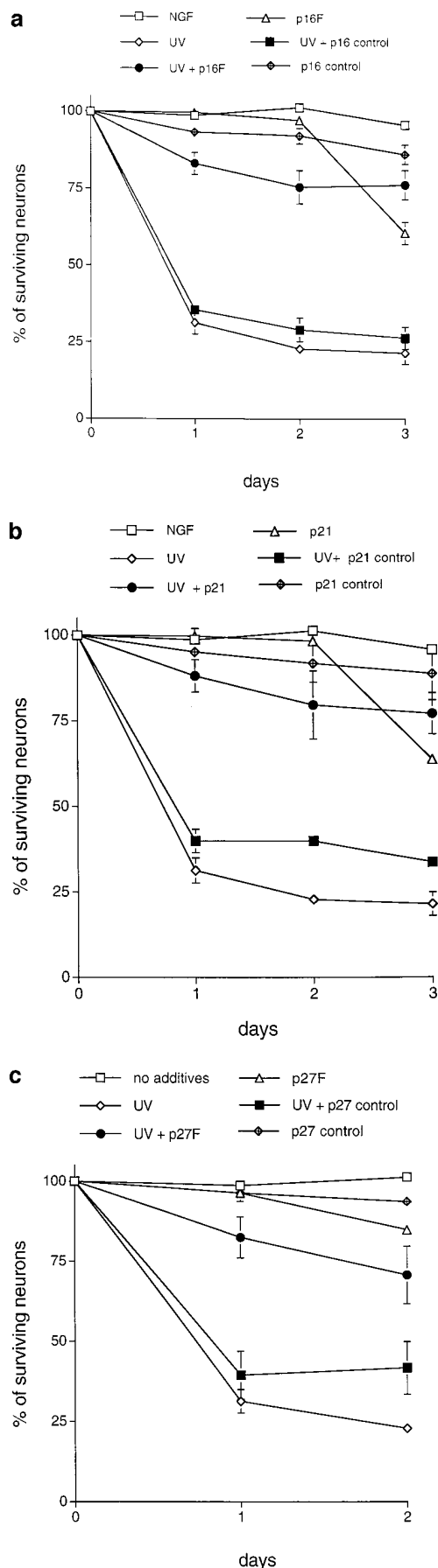
D1-associated kinase activity was performed as previously described (Matsushime et al., 1994). In brief, cortical neurons were treated for various times with camptothecin (10  $\mu\text{M}$ ). The cells were washed twice with cold PBS and harvested in IP buffer as previously described (Matsushime et al., 1994). Cell lysates were then precleared by incubation with 75  $\mu\text{l}$  protein G-agarose beads (Sigma Chemical Co.) for 1 h. 1  $\mu\text{g}$  of anticyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was then added to 300  $\mu\text{g}$  of cell lysate and incubated for 3 h. As control, lysate containing no antibody was used. 50  $\mu\text{l}$  of protein G beads were then added to the lysates and incubated for 1 h. Washing and kinase assay was performed as previously described (Matsushime et al., 1994). pRb (1  $\mu\text{g}$ ; Santa Cruz Biotechnology) was used as substrate. pRb was then resolved on a 10% SDS-polyacrylamide gel and incorporation of  $\text{P}^{32}$  was analyzed by autoradiography and densitometry.

## Results

### CKI Expression Protects Sympathetic Neurons from Death Evoked by UV Irradiation and AraC Treatment

To test the hypothesis that CDK activity is a required element for neuronal apoptosis evoked by DNA-damaging agents, we used Sindbis virus to express CKIs and kinase inactive mutant forms of G1-associated CDKs in sympathetic neurons and evaluated the effects of expression on neuronal death evoked by UV irradiation and AraC treatment. We have previously shown that infection with recombinant Sindbis virus is an efficient means by which to target and express heterologous genes in sympathetic neurons and that the presence of the virus itself does not interfere with the apoptotic pathway (Park et al., 1997b). We first assessed the effects of expression of CKI genes delivered by Sindbis virus on UV-irradiated or AraC-treated sympathetic neurons. Recombinant Sindbis viruses containing the CKIs p16, p21, or p27 were engineered as previously described (Park et al., 1997b). The latter two were tagged with the FLAG epitope at their COOH-terminal ends. In a past study, these behaved comparably to non-

*Figure 1.* Expression of CDK inhibitors using the Sindbis viral delivery system suppresses death of primary cultured sympathetic neurons irradiated with UV. Each point is the mean  $\pm$  SEM of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of UV irradiation. Control viruses for each vector were generated by removal of the start codon and in some cases, introduction of a premature stop site. F, the wild-type protein has a FLAG epitope attached to the COOH terminus. Effects of overexpression of (a) p16, (b) p21, (c) p27 and respective controls on the time course of survival of sympathetic neurons after UV irradiation.



tagged constructs in providing protection from death caused by NGF deprivation (Park et al., 1997b). Control viruses for each CKI-containing Sindbis vector were constructed with the initiation codon deleted and a premature stop codon introduced near the 5' terminus if another methionine was present within 200 bp downstream of the Sindbis virus promoter. We have shown that epitope-tagged p27 and p16 proteins are detected in almost all the neurons after 2 d of infection under the conditions described here (Park et al., 1997b). In addition, we have shown that in neuronal cultures infected with CAT-expressing Sindbis; CAT activity was detectable  $\leq 7$  d after infection (Park et al., 1997b).

Each of the CKI-expressing Sindbis viruses, but not the control "non-coding/stop" recombinant constructs, promoted survival of infected sympathetic neurons exposed to UV irradiation (300 J/m<sup>2</sup>) or AraC (100  $\mu$ M) in the continuous presence of trophic support by NGF. All three CKIs showed comparable survival effects in each paradigm of DNA damage. As shown in Fig. 1, 1 and 2 d after UV irradiation,  $\sim 70$ –80% of the neurons expressing the CKIs were viable as compared with only 30–40% in control irradiated cultures. Protection with the CKIs was also observed for AraC-induced death of sympathetic neurons. 2 d after AraC treatment,  $\sim 50$ –55% of the neurons died in the control cultures whereas only  $\sim 20$ % death was observed for the CKI-expressing neurons (Fig. 2). Neurons rescued by the CKIs displayed phase bright cell bodies typical of viable cells and intact neurites. Uninfected or control virus-infected dying neurons displayed dark apoptotic bodies and degenerating neurites (Fig. 3; data not shown).

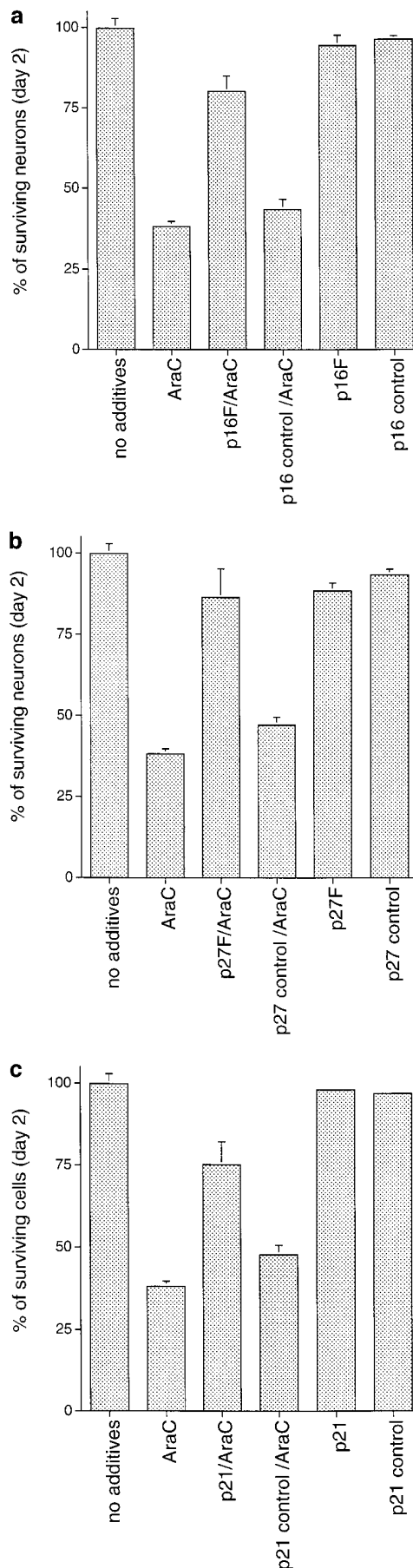
To determine whether the survival-promoting activities we observed were not simply due to heterologous protein expression by the Sindbis virus, we infected neuronal cultures with recombinant viruses expressing either CAT or a control single chain antibody (ScFv). Infection with these viruses had no effect on the rate of death of sympathetic neurons exposed to UV irradiation or AraC treatment (data not shown).

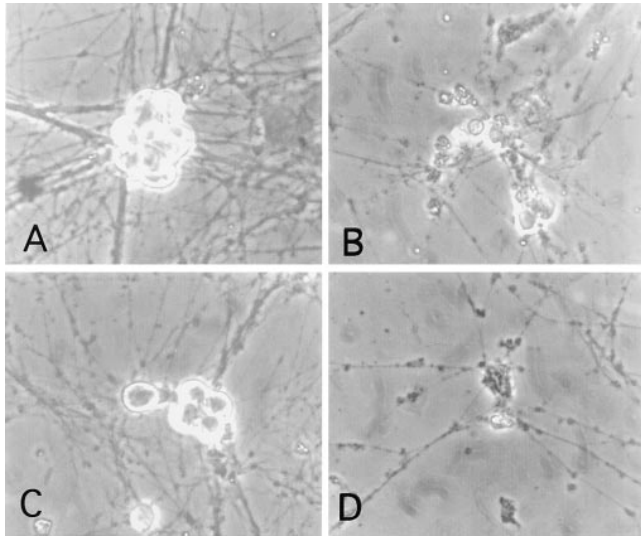
Some degree of cytotoxicity was observed after 3 d in cultures not exposed to DNA-damaging agents and infected with p16-, p21-, or p27-expressing recombinant virus (Fig. 1; data not shown). The reasons for this are unclear.

#### **Expression of Kinase Inactive Cdk4 or Cdk6, but Not Cdk2 or Cdk3, Protects Sympathetic Neurons from UV Irradiation and AraC Treatment**

We next examined whether the effects of the CKIs might be due, at least in part, to effects on CDKs, and if so, to determine which CDKs may play a required role in DNA

*Figure 2.* Expression of CDK inhibitors using the Sindbis viral delivery system suppress death of primary cultured sympathetic neurons treated with AraC. Each point is the mean  $\pm$  SEM of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of AraC treatment. Control viruses for each vector were generated by removal of the start codon and in some cases, introduction of a premature stop site. FLAG, the wild type protein has a FLAG epitope attached to the COOH terminus. Effects of overexpression of (a) p16, (b) p21, (c) p27 and respective controls on the time-course of survival of sympathetic neurons after AraC treatment.





**Figure 3.** Phase-contrast micrographs of rat sympathetic neurons maintained in NGF-containing medium and treated for 2 d with the following: (a) no additives, (b) UV, (c) p16-Sindbis virus + UV, and (d) p16 control-Sindbis virus + UV.

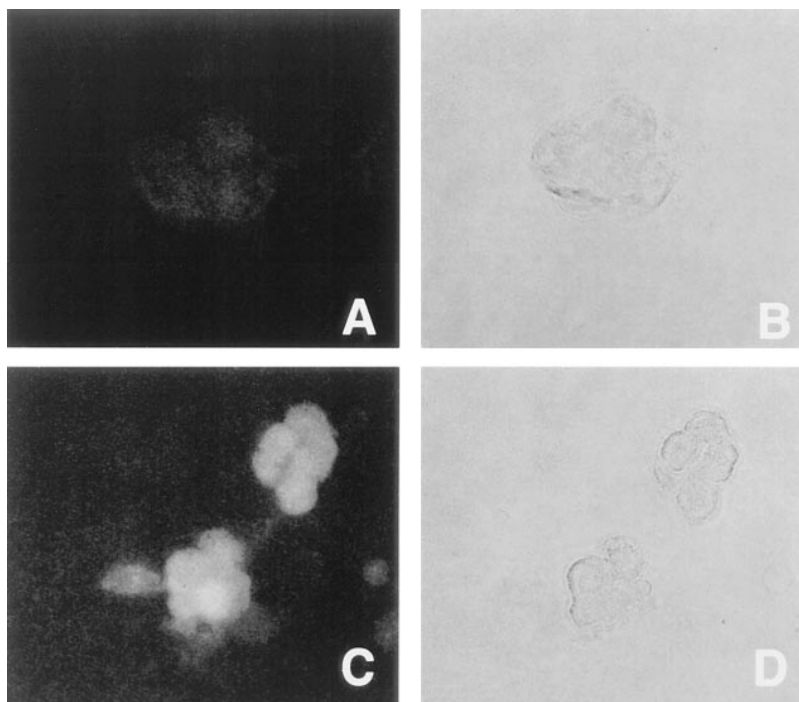
damage-induced neuronal death. Previous pharmacologic studies suggested that neuronal death evoked by DNA-damaging agents requires the participation of CDKs involved in G1 to S progression (Park et al., 1997a, 1998). Consequently, we constructed recombinant Sindbis virus-expressing kinase inactive forms of Cdk2, Cdk3, Cdk4, and Cdk6 (all with or without FLAG tags). These point mutants were first described by van den Heuvel and Harlow (1993) and appear to act as DN (see Discussion). Cdks 2 and 3 are reported to be associated with G1 progression of actively dividing cells (van den Heuvel and Harlow, 1993); whereas Cdks 4 and 6 appear to control entry into S phase

of cells emerging from quiescence (Doree and Galas, 1994; Diehl and Sherr, 1997; Connell-Crowley et al., 1998). Neuronal expression of each DN CDK was confirmed by immunofluorescent localization of the FLAG epitope. This revealed that essentially all neurons expressed the epitope after infection and that there was a degree of variation of level of expression from cell to cell. Expression of the DN CDKs was detected up to at least 7 d after infection (Fig. 4; data not shown). Sindbis virus-mediated expression of DN Cdk4 or 6 significantly delayed death of neurons exposed to UV irradiation. 2–4 d after irradiation, ~80–90% survival was observed for cultures infected with virus expressing DN Cdk4/6, whereas those neurons that were uninfected or infected with control virus showed only ~25–35% survival (Fig. 5, c and d). In contrast, infection with Sindbis virus-expressing DN Cdk2 or Cdk3 had no effect on survival of UV-irradiated neurons (Fig. 5, a and b).

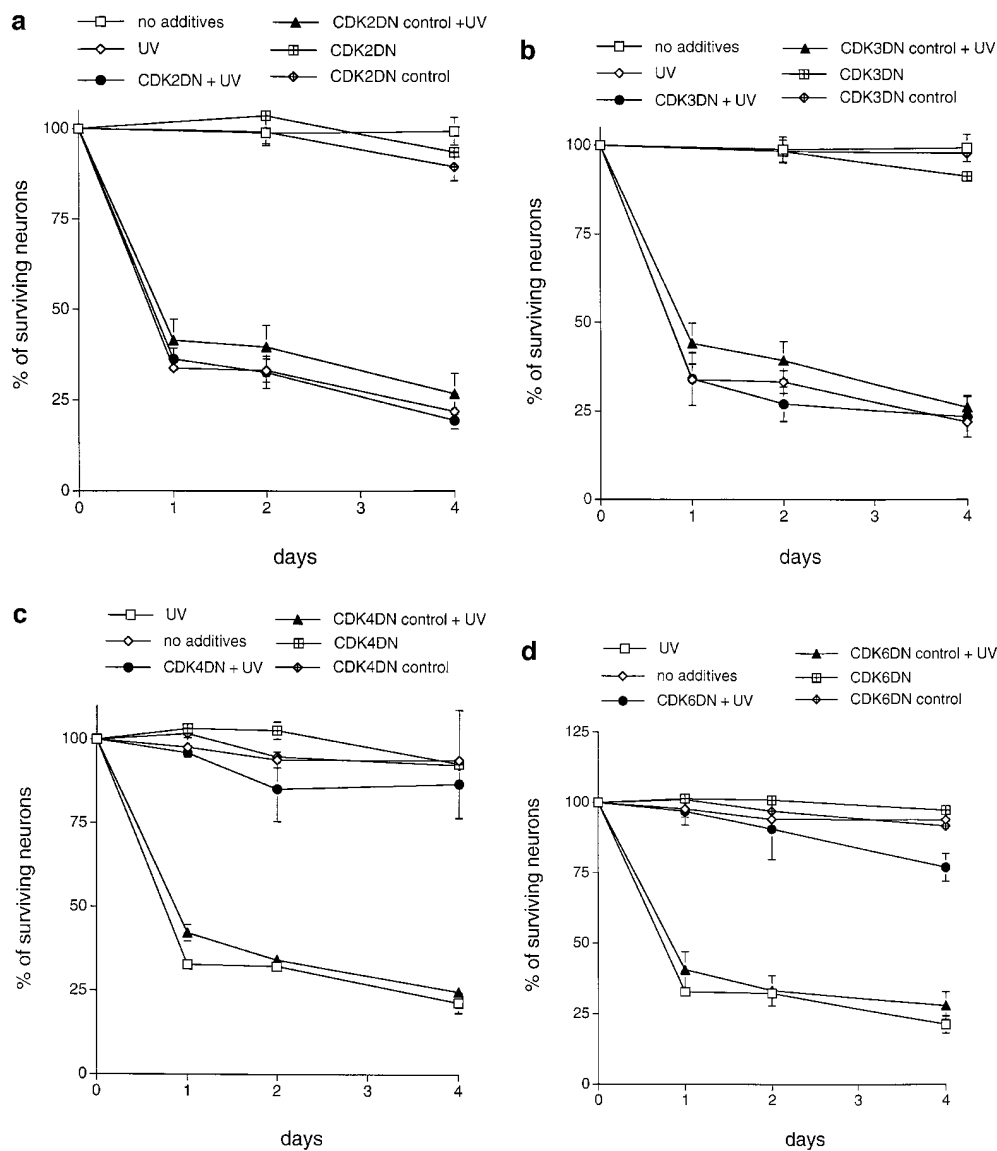
Expression of DN Cdk4 and 6 significantly delayed the death of AraC-treated sympathetic neurons (~80–85% survival at day 2 with DN Cdk4/6 versus ~50% survival in cultures infected with control viruses or uninfected; Fig. 6, c and d). Unlike the case of UV treatment, however, survival of AraC-treated cells fell beyond 2 d in cultures expressing DN Cdk4/6 expression. The reason for this is unclear but one possibility is that continual exposure to AraC may overwhelm the abilities of the DN CDKs to inhibit the presumed continual generation of apoptotic signals. Again, DN Cdk2 or 3 expression had no effect on death of AraC-treated sympathetic neurons (Fig. 6, a and b). The appearances of neurons rescued by DN Cdk4/6 expression is similar to those rescued by the CKIs.

#### **Expression of p16 and p27 and DN Cdk4/6 but Not DN Cdk2/3 Protects Cortical Neurons from Camptothecin-induced Death**

Given the protection of sympathetic neurons from DNA-



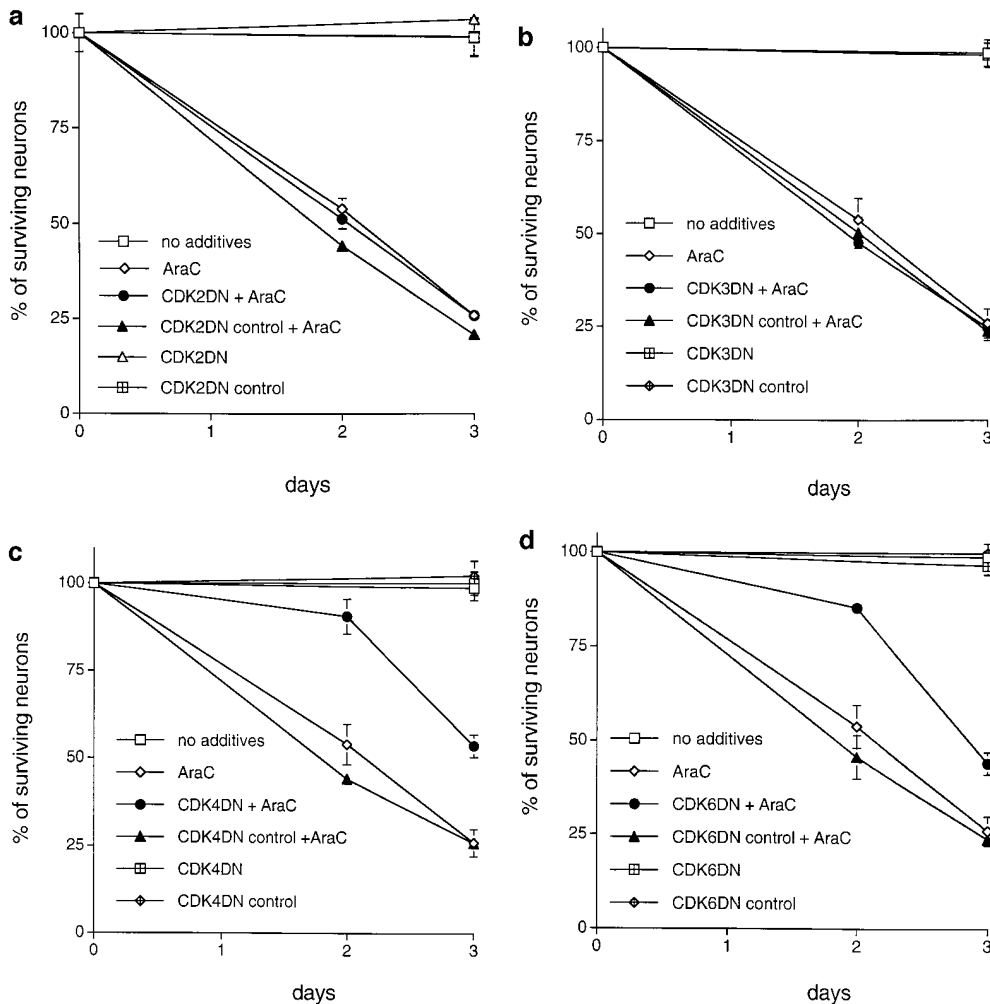
**Figure 4.** Immunofluorescence (a, c) staining with an antibody directed against the FLAG epitope or corresponding light micrographs (b, d) of sympathetic neurons in culture infected with Sindbis virus-expressing Cdk3DN-FLAG (c, d) or containing the Cdk3 control “stop” sequence (a, b). Neurons were stained seven days post-infection.



**Figure 5.** The effects of expression of DN CDKs on survival of cultured UV-irradiated sympathetic neurons. Each point is the mean  $\pm$  SEM of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of UV irradiation. Control viruses for each vector were generated by removal of the start codon and in some cases, introduction of a premature stop site. Effects of overexpression of (a) Cdk2DN, (b) Cdk3DN, (c) Cdk4DN, (d) Cdk6DN and respective controls on the time course of survival of UV-irradiated sympathetic neurons.

damaging agents provided by expression of CKIs and DN Cdk4/6, we extended our observations to neurons of the central nervous system. Cultures highly enriched in cortical neurons were obtained from 18-d-old rat embryos and death was induced by exposure to the topoisomerase inhibitor camptothecin (10  $\mu$ M; Morris and Geller, 1996). Within 1 d, >90% of the neurons were killed by this drug. Past studies suggest that such death is due to transcriptionally mediated DNA damage formation (Morris and Geller, 1996; Park et al., 1997a; Morris, E.J., D.S. Park, J.C. Dreixler, L.A. Greene, and H.M. Greene. 1997. *Soc. Neurosci. Abstr.* 23:881). Experiments were carried out with cultures infected with the variety of recombinant Sindbis viruses described above for 1 d before treatment and for an additional 24 h in the presence of 10  $\mu$ M camptothecin. Immunofluorescence studies on cultures infected with virus-expressing FLAG-tagged DN Cdk3 and Cdk6 revealed expression in almost all neurons (Fig. 7; data not shown). No expression of CDKs was observed in cultures infected with “stop” control viruses. In addition, Western

blot analyses of these cultures verified that the various FLAG-tagged DN CDKs were expressed at similar levels (Fig. 8 a). Blotting also confirmed the expression of p16 and p27 (Fig. 8 b). For unknown reasons, the virus-containing FLAG-tagged p21 failed to yield detectable levels of expression in cortical neurons and so was not further pursued. Control cultures exposed to the viruses and not exposed to camptothecin showed a modest (25–35%) loss of viability at 24 h. Also, most control viruses showed a small, but consistent survival-promoting effect on camptothecin-treated cortical neurons (10–25% survival) when compared with uninfected (4% survival) cultures (Fig. 9). Irrespective of these background effects, as shown in Fig. 9, p16, p27, DN Cdk4, and DN Cdk6 conferred significant protection from camptothecin treatment. Survival in these cases was nearly equal to that observed in virus-treated cultures that lacked camptothecin exposure (60–70%) whereas control “stop” viruses resulted in only 10–20% survival in response to camptothecin treatment. Little or no difference in survival, however, was observed when the



**Figure 6.** The effects of expression of DN CDKs on survival of cultured sympathetic neurons treated with AraC. Each data point is the mean  $\pm$  SEM of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of AraC treatment. Control viruses for each vector were generated by removal of the start codon and in some cases, introduction of a premature stop site. Effects of overexpression of (a) Cdk2DN, (b) Cdk3DN, (c) Cdk4DN, (d) Cdk6DN and respective controls on the time course of survival of AraC-treated sympathetic neurons.

cortical cultures were infected with DN Cdk2 or Cdk3 or the CAT/Sfcv controls (Fig. 9 b; data not shown).

### *Cyclin D1-associated Kinase Activity Is Markedly Elevated in Camptothecin-treated Cortical Neurons*

The observations that DN Cdk4 and 6 protect from neuronal death induced by DNA damage suggest that the activities of the corresponding endogenous CDKs should be elevated in dying neurons. To test this, we measured cyclin D1-associated kinase activity in extracts of cortical neurons that had been treated for various times with camptothecin. As shown in Fig. 10, we observed a sevenfold increase in cyclin D1-associated kinase activity by 1 h of treatment. This increase in activity peaked at  $\sim$ 10-fold by 2–4 h, and was somewhat diminished at 8 h.

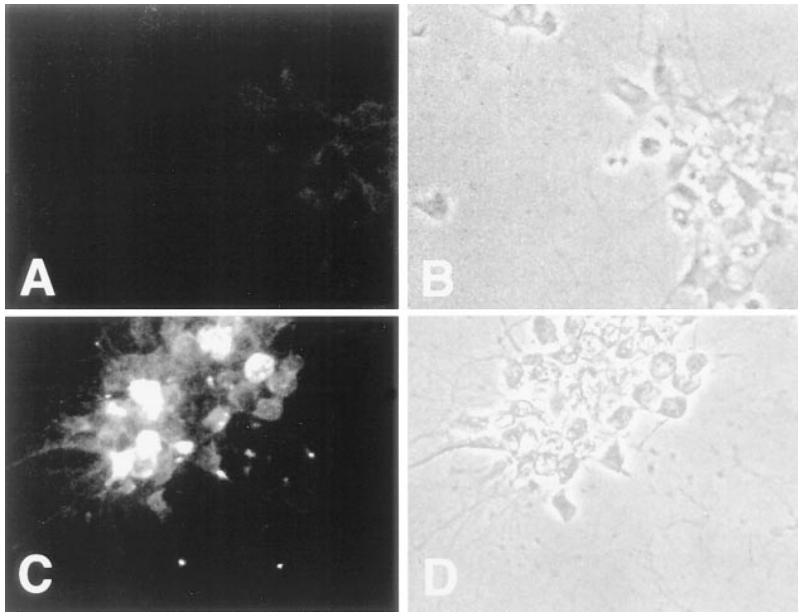
## **Discussion**

### *Sindbis Viruses as a Gene Delivery System to Evaluate Neuronal Death Resulting from DNA-damaging Agents*

The Sindbis virus represents a potentially useful and efficient method for targeting heterologous genes to primary postmitotic neurons. We have previously demonstrated

that the Sindbis virus vector efficiently targets sympathetic neurons and can be used to evaluate the mechanisms of death resulting from NGF deprivation. A variety of control viruses either expressing non-death-related proteins or with premature stop codons inserted into the heterologous genes of interest had minimal effect on the kinetics of death of sympathetic neurons deprived of NGF (Park et al., 1997b). We now show that such control viruses have minimal effects on the kinetics of death of sympathetic neurons exposed to the DNA-damaging agents UV irradiation and AraC. These findings thus indicate that the Sindbis virus is also an effective system for evaluating the effects of exogenous genes in sympathetic neuron death evoked by DNA damage.

We show here that, as with sympathetic neurons, cultured primary central nervous system (CNS) neurons are also efficiently targeted by Sindbis viruses. However, unlike the case of sympathetic neurons, with camptothecin-treated cortical neurons, there is a small but significant protective effect of the control viruses themselves. The viruses also somewhat diminished background survival after 48 h in control cortical cultures. Nevertheless, our analyses revealed significant differences between responses to control viruses and to those expressing certain genes related to cell cycle. Moreover, there was good correspondence



**Figure 7.** Immunofluorescence staining with an antibody directed against the FLAG epitope (*a*, *c*) or corresponding light micrographs (*b*, *d*) of cortical neurons in culture infected with Sindbis virus expressing Cdk3DN-FLAG (*c*, *d*) or containing the Cdk3 control sequence (*a*, *b*). Neurons were stained 1 d after infection.

between those constructs that were effective in protecting sympathetic neurons and in protecting cortical neurons. Accordingly, with appropriate controls, it appears that the Sindbis virus is a convenient vector for at least short-term gene targeting to CNS as well as to PNS neurons in culture.

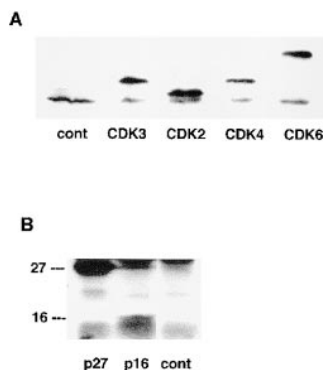
#### **Cell Cycle and Neuronal Death Resulting from DNA-damaging Agents**

Observations that postmitotic neurons, like proliferating cells, are vulnerable to DNA topoisomerase-I inhibitors such as camptothecin, chain terminators such as AraC, and ionizing radiation led to the suggestion that such agents evoke neuronal death by causing DNA damage (Morris and Geller, 1996; Park et al., 1997*a*). In dividing cells, these DNA-damaging agents induce and activate CKIs and the tumor suppressor p53, which acts to regulate cell cycle progression (Sherr, 1996). This, in turn, raised the hypothesis that DNA damage may by some means activate elements of the cell cycle machinery in neurons that would participate in activation of an apoptotic pathway (Park et al., 1997*a*). This was supported by observations that non-peptide G1/S blockers and CDK inhibitors suppress the death of neuronally differentiated PC12 cells,

sympathetic neurons, and cortical neurons exposed to UV irradiation, camptothecin, or AraC (Park et al., 1997*a*). In light of these findings, we undertook the present experiments to (*a*) confirm the previous pharmacological evidence implicating CDKs in neuronal death induced by DNA-damaging agents and (*b*) identify the particular CDKs that might be involved.

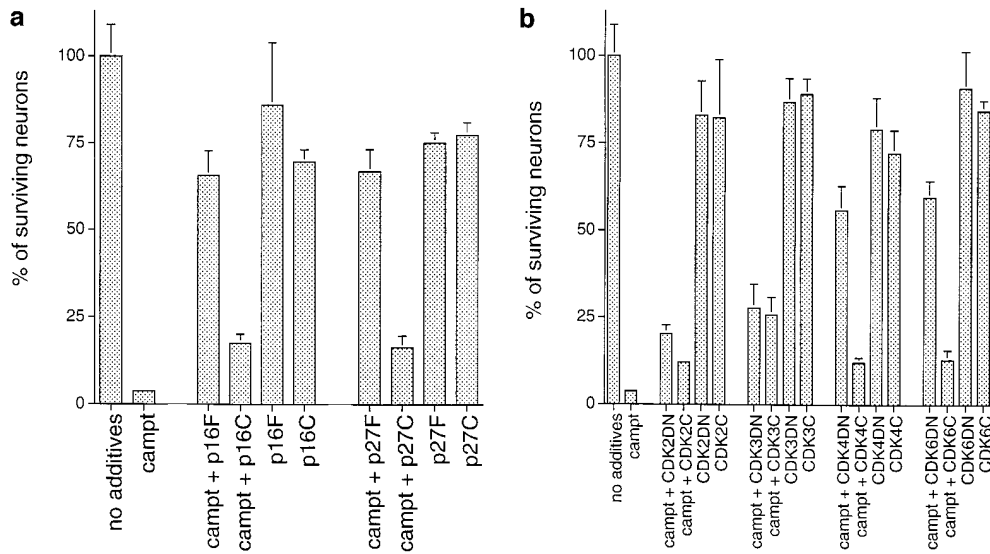
We report here that death of sympathetic neurons exposed to UV irradiation or AraC can be inhibited by the CKIs p16, p21, and p27, and that death of cortical neurons exposed to camptothecin can be inhibited by p16 and p27. In addition to its CDK inhibitory activity, p21 is reported to bind to PCNA and to block c-Jun kinase activity, thus posing alternative means by which it may interfere with death (Shim et al., 1996). However, p16 does not inhibit c-Jun kinase (Shim et al., 1996) and this, coupled with the protective actions of DN Cdk4 and Cdk6, favors a mechanism in which the various CKIs we used suppress death by virtue of their abilities to interfere with CDK activities.

To extend our experiments with CKIs, we expressed in neurons various CDKs that contained an Asp to Asn mutation in the conserved KLADFGRLAR kinase consensus domain. Although catalytically inactive, such mutants should retain their capacity to bind cyclins and hence to act as DN. van den Heuvel and Harlow (1993) demonstrated that the mutant forms of Cdk2 and Cdk3 block progression of actively dividing cells, apparently by acting as DN antagonists for endogenous CDKs. In the latter study, the mutated forms of Cdk4 and Cdk6 did not block proliferation. However, this lack of action may be due to the finding that Cdk4-cyclin D1 activity may play a more important role in controlling S phase entry for cells emerging from quiescence rather than for cells in a state of active proliferation (Doree and Galas, 1994; Diehl and Sherr, 1997; Connell-Crowley et al., 1998). Although we have not yet been able to directly test whether the mutated Cdk4 and Cdk6 constructs act as DN in neurons, recent cotransfection-based assays in SAOS-2 and MEF7 cells show that these mutants block the ability of overexpressed



**Figure 8.** Western blot analyses of cortical neurons expressing FLAG-tagged DN CDK2/3/4/6 and p27 (*a*) and p27 and p16 (*b*). *cont*, control samples in which the neurons were infected with a control virus. The blots were analyzed using an anti-FLAG antibody. Neurons were lysed after 24-h infection with the appropriate viruses.





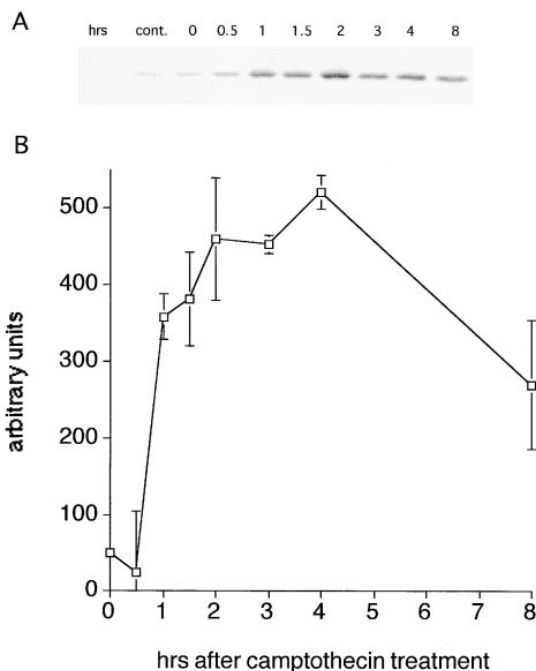
**Figure 9.** Effects of expression of CDK inhibitors (*a*) or DN CDKs (*b*) on survival of primary E18 cultured rat cortical neurons treated with camptothecin (*campt*). Each value is the mean  $\pm$  SEM of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of camptothecin treatment.

cyclin D1 to reverse Rb-mediated repression of E2F activity (S.P. Chellappan, personal communication). The most likely interpretation of these observations is that the mutant Cdk4 and Cdk6 constructs act as DN to suppress formation of active complexes between their corresponding endogenous forms and cyclin D1. Taken together, the above points strongly support the likelihood that the kinase inactive CDK mutants we used act as DN.

Our findings revealed that expression of mutated Cdk4

and 6, but not Cdk2 or Cdk3 effectively suppressed death in our paradigms of DNA damage. In this light, it is significant that the one set of CDKs that are blocked in common by the three CKIs we used are Cdk4 and Cdk6 (Sherr and Roberts, 1995). Our findings that cyclin D1-associated kinase activity is rapidly elevated in response to DNA damage of neurons are also consonant with a role for Cdk4/6 in the pathway by which DNA-damaging agents cause death of neurons. Finally, our observations are consistent with the reports of the presence of Cdk4 but not Cdk2 transcripts in sympathetic neurons (Freeman et al., 1994) and our own findings of the presence of Cdk4 in cortical neurons (Park, D.S., unpublished results). It is also interesting that DN Cdk3 failed to protect neurons from DNA damage, although Cdk3 has been shown to be required for cell cycle progression in proliferating cells (van den Heuvel and Harlow, 1993). It is unknown, however, whether Cdk3 is present in neurons or whether it is activated during the death process. Finally, we have previously shown that the pharmacological CDK inhibitors olomoucine and flavopiridol protect sympathetic neurons and PC12 cells from death resulting from NGF deprivation and DNA damage (Park et al., 1996a, 1997a). However, while flavopiridol inhibits Cdk4 activity, olomoucine does not (Vesely et al., 1994). This suggests that additional CDKs may also be important for regulation of neuronal death.

The potential function of Cdk4/6 action in death of sympathetic and cortical neurons treated with DNA-damaging agents is unclear. One well-studied substrate of Cdk4/6 is the retinoblastoma protein (pRB). pRB, the product of the retinoblastoma tumor suppressor gene, appears to play a role in regulating the G1 phase of the cell cycle (Chellappan et al., 1991; Weinberg, 1995). pRB binds to and inhibits the transcription factor E2F and its phosphorylation by Cdk4/6 suppresses this interaction, thereby permitting E2F activation. Interestingly, expression of pRB in multiple cellular contexts, including neurons, is protective against apoptosis (Berry et al., 1996; Fan et al., 1996; Macleod et al., 1996), while loss of pRB interaction facilitates death (Liu and Kitsis, 1996; Shan et al., 1996). Also, pRB



**Figure 10.** Cyclin D1-associated kinase activity is elevated in cortical neurons treated with camptothecin. Cyclin D1-associated kinase activity was calculated by measuring the incorporation of P32 onto pRB substrate and subtracting the control background value. Each point is the mean  $\pm$  SEM of data from three separate experiments. (A) An example autoradiogram showing the incorporation of P32 into pRB.

knockout mice display excess developmental neuronal cell death (Jacks et al., 1992; Macleod et al., 1996) and overexpression of E2F evokes death in a variety of cell types (Qin et al., 1994; Hiebert et al., 1995). Accordingly, Cdk4/6 may act in DNA damage-evoked neuronal death to phosphorylate pRB thereby altering its functional state and leading, among other potentially lethal events, to dysregulation of E2F activation. In support of this, preliminary evidence suggests that pRB is hyperphosphorylated and degraded in camptothecin-treated cortical neurons (Park, D.S., and E.J. Morris, unpublished results) and a recent report indicates that pRB becomes hyperphosphorylated during cisplatin-induced death of sensory neurons with concomitant increase in Cdk4 and cyclin D1 levels (Gill and Windebank, 1998).

Neuropathies constitute a significant clinical problem in the therapeutic administration of anticancer agents that appear to work by damaging DNA. For example, AraC, which is commonly used in treatment for leukemias, can cause cerebellar neuropathies (Winkelman and Hines, 1983). In addition, radio- and chemotherapy for brain neoplasms results in dose-limiting acute and delayed toxicity leading to edema, seizures, neuropsychiatric deterioration, IQ decrements, and death (Levin et al., 1993). Our findings suggest a potential mechanism by which DNA-damaging agents can cause degeneration of postmitotic neurons and specifically point to cell cycle components. Such knowledge, in turn, may lead to palliative treatments, perhaps through administration of inhibitors of specific CDKs.

### **Comparison between Death Signaling Mediated by DNA Damage, Oxidative Stress, and NGF Deprivation**

Several lines of evidence have implicated cyclins and CDKs in apoptosis of neuronal cells caused by withdrawal of trophic support. For example, NGF deprivation leads to increased Cdc2 activity and cyclin B expression in neuronal PC12 cells (Brooks, S.F., L.A. Gibson, and L.L. Rubin. 1993. *Soc. Neurosci. Abstr.* 19:885; Gao and Zalenka, 1995); expression of the CKI p21 is required for survival of differentiated neuroblastoma cells (Poluha et al., 1996) and CKI p16 protects neuroblastoma cells from death caused by trophic factor deprivation (Kranenburg et al., 1996). In addition, we have previously shown that expression of CKIs or DN Cdk4/6 protects sympathetic neurons against NGF deprivation (Park et al., 1997b).

Thus with respect to the involvement of CDKs, our present experimental findings suggest parallel mechanisms of cell death evoked by NGF withdrawal and exposure to DNA-damaging agents. However, our previous evidence has indicated that the death evoked by these stimuli is not identical (Park et al., 1998). In particular while death resulting from DNA damage does require induction of caspase activity, the caspase(s) involved is different than that mediating death by way of NGF deprivation (which requires activation of Nedd-2/caspase 2; Troy et al., 1997) or to oxidative stress induced by superoxide dismutase 1 depletion (which requires ICE or ICE-like activity; Troy et al., 1996). Moreover, death caused by oxidative stress is not prevented by CDK inhibitors nor is that triggered by oxidative stress or DNA-damaging agents blocked by the

presence of NGF (Park et al., 1998). Consequently, it can be anticipated that in addition to CDKs, additional pathway-specific elements must distinguish the mechanism by which DNA-damaging agents trigger neuronal apoptosis.

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