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Regulation of Neuronal Survival and Death by E2F-Dependent Gene Repression and Derepression

David X. Liu[1] and Lloyd A. Greene
Department of Pathology
Taub Center for Alzheimer's Disease Research and
Center for Neurobiology and Behavior
Columbia University College of
Physicians and Surgeons
New York, New York 10032

Summary

Neuronal death induced by a variety of means requires participation of the E2F family of transcription factors. Here, we show that E2F acts as a gene silencer in neurons and that repression of E2F-responsive genes is required for neuronal survival. Moreover, neuronal death evoked by DNA damaging agents or trophic factor withdrawal is characterized by derepression of E2F-responsive genes. Such derepression, rather than direct E2F-promoted gene activation, is required for death. Among the genes that are derepressed in neurons subjected to DNA damage or trophic factor withdrawal are the transcription factors B- and C-myb. Overexpression of B- and C-myb is sufficient to evoke neuronal death. These findings support a model in which E2F-dependent gene repression and derepression play pivotal roles in neuronal survival and death, respectively.

Introduction

Neuronal apoptosis is an integral part of nervous system development (Oppenheim, 1991) and plays a role in neuronal injury and disease (Mattson, 2000). A growing body of evidence has implicated cell cycle regulators as required mediators for neuronal death evoked by treatments as diverse as neurotrophic factor deprivation (Park et al., 1997a), DNA damage (Park et al., 1997b, 1998), exposure to β-amyloid (Giovanni et al., 1999), withdrawal of elevated K^+ (Padmanabhan et al., 1999), and stroke (Osuga et al., 2000). Altered neuronal expression/activity of cell cycle regulators has also been correlated with several neurodegenerative disorders (Zhu et al., 1999). However, the molecular basis for the involvement of cell cycle elements in neuronal apoptosis is unclear.

A number of the cell cycle molecules that have been associated with neuronal death converge on regulation of the E2F family of transcription factors. Functional E2F transcription factors are heterodimers of E2F and DP proteins, and their activities are also regulated by the Rb "pocket" protein family (Chellappan et al., 1991; Dyson, 1998). During the G1 phase of the cell cycle, pocket proteins are in a non- or hypophosphorylated state that inhibits E2F-promoted gene activation and that permits E2F sites to act as silencing elements (Harbour and Dean, 2000). Upon mitotic stimulation, pocket proteins become phosphorylated by cyclin-dependent kinases (CDKs), which results in expression of a variety of E2F-responsive genes (Muller et al., 2001). CDK4/6 becomes active before CDK2 (Lundberg and Weinberg, 1998) and appears to phosphorylate Rb in mid-G1. This first step of phosphorylation induces an intramolecular change in Rb, which in turn abolishes its active repression of E2F-responsive genes and uncovers the sites for CDK2 phosphorylation (Harbour et al., 1999; Zhang et al., 2000). The subsequent phosphorylation of Rb at the end of G1 by CDK2-cyclin E complexes promotes the release of Rb from E2F and consequent E2F-dependent activation of S phase genes (Harbour et al., 1999). Therefore, CDK4/6 and CDK2 complexes are activated and promote cell proliferation by phosphorylation of Rb family proteins in a progressive and sequential manner that includes both gene derepression and transactivation.

Cell cycle regulators, especially G1 checkpoint molecules, have been found to be deregulated in diseased neuronal tissue and apoptotic neurons (Liu and Greene, 2001). For example, elevated levels of cyclin D1 mRNA or protein are detected in vulnerable neurons following induced ischemia (Guegan et al., 1997) and in neuroblastoma cells (Kranenburg et al., 1996) and sympathetic neurons under trophic withdrawal (Freeman et al., 1994). Furthermore, CDK4 and CDK2 are elevated in neurons of patients with Alzheimer's disease (McShea et al., 1997; Zhu et al., 1999), and multiple lines of evidence suggest attempted reentry into cell cycle as a proximal pathophysiology in AD (Husseman et al., 2000; Raina et al., 2000). However, these observations say little about how cell cycle proteins work to regulate death.

Two neuronal death systems in which the role of specific cell cycle molecules has been studied in depth are that of neurons deprived of trophic support or subjected to DNA damaging agents such as UV irradiation or the topoisomerase I inhibitor camptothecin. Multiple lines of evidence support required roles for cyclin D1 and CDK4/6 activity in these neuronal death paradigms (Courtney and Coffey, 1999; Park et al., 1996a, 1997a, 1997b, 1998, 2000). For instance, exposure of cortical neurons to DNA damaging agents elicits a dramatic increase of cyclin D-associated kinase activity that is followed by hyperphosphorylation and subsequent loss of Rb family members (Park et al., 1998, 2000). In addition, expression of D/N CDK4/6 but not D/N CDK2 blocks neuronal apoptosis caused by DNA damage or NGF withdrawal (Park et al., 1997a, 1998). Taken together, these observations support the notion that a cyclin D-CDK 4/6-Rb-E2F pathway is a major component of the apoptotic mechanism in neurons subjected to trophic factor deprivation or DNA damage.

How might CDK 4/6-dependent modification/loss of E2F-pocket protein complexes lead to neuronal apoptosis? Death of neurons in many circumstances, including DNA damage and trophic factor withdrawal, requires gene transcription (Freeman et al., 1994; Morris and Geller, 1996). In this light, the modification/loss of E2F-pocket protein complexes in DNA-damaged neurons raises two potential mechanisms for changed ex-

[1] Correspondence: dl345@columbia.edu

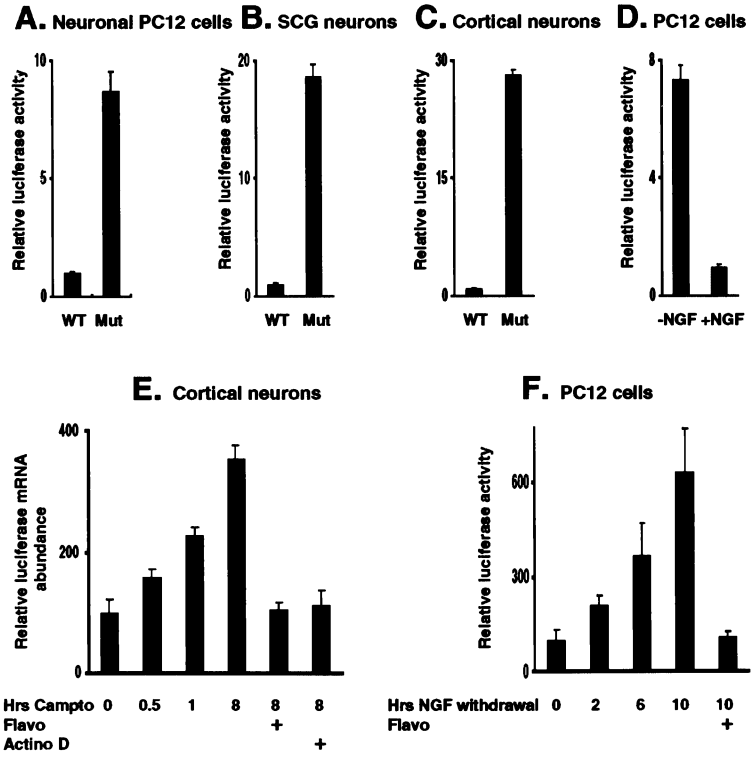

Figure 1. E2F Sites in the B-myb Promoter Serve as Potent Transcriptional Repressors in Neuronal Cells and This Repression Is Relieved in Response to Camptothecin and NGF Withdrawal

(A–C) Activity of luciferase reporter constructs driven by the wild-type B-myb promoter and a mutant B-myb promoter in which the E2F site is compromised by a point mutation. Neuronal PC12 cells, sympathetic neurons, and cortical neurons were transfected as described in Experimental Procedures, with Bmyb-luc or Bmyb(Mut)-luc and pcDNA-LacZ. Later (48 hr), cells were harvested for luciferase and β-gal assays. Results are reported in relative light units and normalized to β-gal activity. Data are means ± SEM (n = 3).

(D) Comparison of Bmyb-luc reporter activity in transfected naive and neuronal PC12 cells. Transfection and reporter assays were carried out as in (A). Data are means ± SEM (n = 3).

(E) Derepression of Bmyb-luc in cortical neurons treated with camptothecin. Transfection was carried out as above, and, 2 days later, cells were exposed to camptothecin for the indicated times and harvested for total RNA. When used, flavopiridol and actinomycin D were added to the cultures 30 min before camptothecin. Expression of luciferase transcripts was quantified by real-time PCR. Results were normalized to LacZ mRNA. Data are means ± SEM (n = 3).

(F) NGF deprivation evokes derepression of Bmyb-luc in neuronal PC12 cells. Transfection of the reporter was carried out as in Experimental Procedures, and, 2 days later, cultures were washed free of NGF and exposed to anti-hNGF for the indicated time. When used, flavopiridol was added to the cultures at the time when anti-hNGF was added into the medium. Reporter activity was determined as in (A). Data are means ± SEM (n = 3).

pression of apoptotic genes: upregulation of death-associated genes by E2F activation and derepression of proapoptotic genes normally held in check by E2F-pocket protein complexes. The purpose of the present study, therefore, was to determine the roles of E2F-dependent gene activation, repression, and derepression in the survival and death of neurons. Our findings indicate that neuronal survival requires active gene repression by E2F-pocket protein complexes. Moreover, we show that derepression but not transactivation of E2F-responsive genes is required for neuronal death induced by DNA damage or NGF withdrawal.

Results

Gene Repression by E2F in Postmitotic Neurons

In replication-competent cells, E2Fs repress their target genes in G1 and induce their expression in G1/S and S (Dyson, 1998). Because postmitotic neurons are permanently in G0/G1, we first determined whether the E2F sites in such cells are in a repressive state. To this end, we transfected neuronal cells with an E2F reporter construct Bmyb-luciferase (Bmyb-luc) or with a control construct [Bmyb(mut)-luc] containing a mutation that abolishes E2F binding and measured the luciferase activity. *B-myb* has been shown to be repressed in G1 and derepressed in S in an E2F-dependent manner (Lam and Watson, 1993). In this and subsequent experiments, we used three different neuronal culture systems: neuronally differentiated PC12 cells, postnatal superior cervical ganglion (SCG) sympathetic neurons, and embryonic cortical neurons. As shown in Figures 1A–1C, the reporter with the mutated E2F site exhibited 8-, 18-, and 27-fold greater activity in neuronal PC12 cells, sympathetic neurons, and cortical neurons, respectively, than its counterpart with an intact E2F binding site. We obtained similar findings with two additional pairs of luciferase reporters with intact and mutated E2F binding sites [cdc25A-luc/cdc25A(mut)-luc; Chen and Prywes, 1999; and DHFR-luc/DHRF(mut)-luc; Slansky et al., 1993; data not shown].

To ascertain whether repression mediated by E2F in neuronal cells reflects their postmitotic state, we also compared expression of Bmyb-luc in naive and neuronal PC12 cells. The former cells are in an active proliferating state, whereas the latter leave the cell cycle in response to NGF treatment (Greene and Tischler, 1976). As shown in Figure 1D, activity in the naive, proliferating cells was 7-fold higher than in neuronal cells. This ratio is similar to that for the mutant versus wild-type reporter in the NGF-treated cultures. Thus, taken together these results indicate that E2F binding sites mediate potent repression of E2F-responsive genes in neurons and neuronal PC12 cells and that this is associated with their postmitotic state.

E2F Repression Is Relieved in Neurons after Exposure to DNA Damaging Agents

Exposure of neurons to DNA damaging agents causes a rapid activation of cyclin D-associated kinase activity

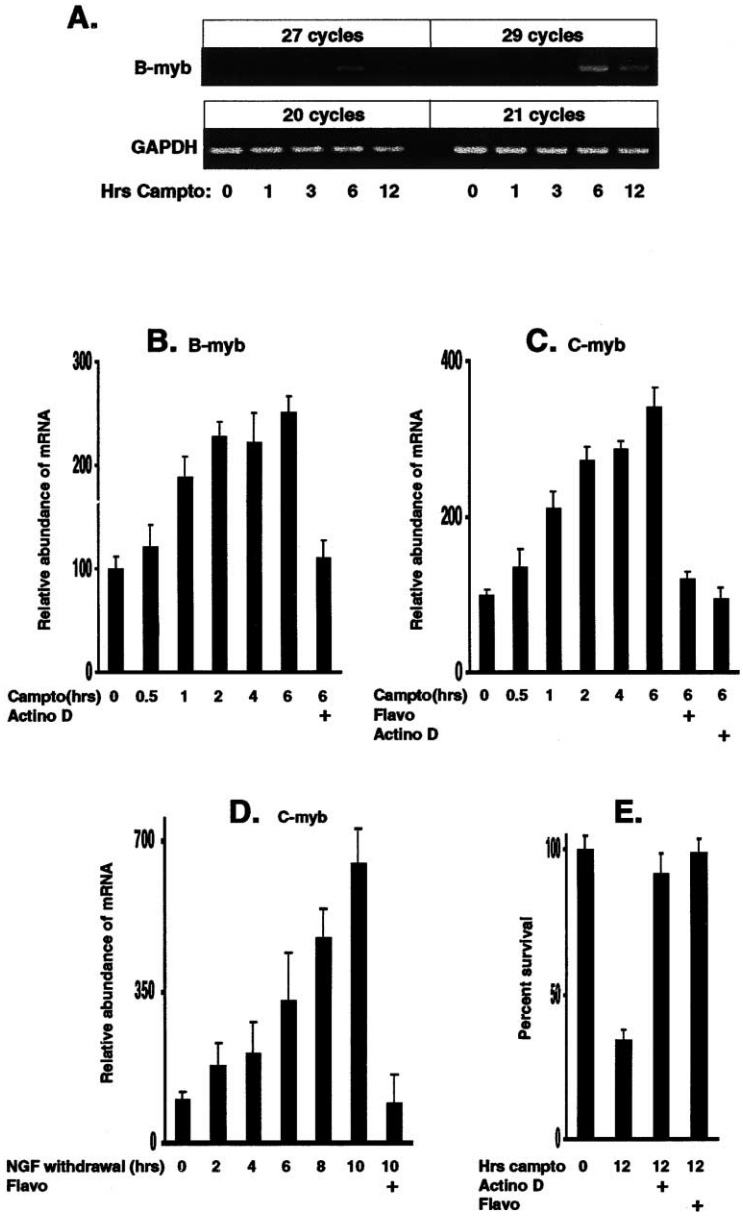

Figure 2. Upregulation of B-myb and C-myb mRNAs in Response to Camptothecin or NGF Withdrawal

(A) Semiquantitative PCR analysis of B-myb mRNA. Total RNA was harvested from cortical neurons treated with camptothecin for the indicated times and converted to cDNA. PCR reactions were performed 27 and 29 cycles for B-myb and 20 and 21 cycles for GAPDH.

(B and C) Increase of endogenous B-myb and C-myb mRNAs in cortical neurons in response to camptothecin treatment. Quantitative real-time PCR measurements of B-myb and C-myb mRNA levels were carried out as described in Experimental Procedures. Expression levels were normalized against GAPDH mRNA. When used, flavopiridol and actinomycin D were added to the cultures 30 min before camptothecin. Data are means ± SEM (n = 3).

(D) NGF withdrawal leads to elevation of endogenous C-myb. Neuronally differentiated PC12 cells were deprived of NGF for the indicated times and assessed for levels of C-myb mRNA as in (B). Data are means ± SEM (n = 3).

(E) Flavopiridol and actinomycin D prevent death of cortical neurons induced by camptothecin. Flavopiridol and actinomycin D were added to cultures 30 min before camptothecin. The percentage of surviving neurons was determined 12 hr later by strip counting. Experimental points are expressed relative to the numbers of cells present before treatment and are reported as means ± SEM (n = 3).

as well as hyperphosphorylation and loss of pocket proteins (Park et al., 1998, 2000). This also leads to loss of E2F-Rb family complexes as judged by gel shift assays (data not shown). We reasoned that the E2F-dependent gene repression present in healthy neurons may therefore be compromised in response to DNA damage. To test this possibility, we assessed the relative levels of luciferase transcripts in neurons transfected with the Bmyb-luc reporter and exposed to camptothecin for various times. The data in Figure 1E indicate that camptothecin promotes a rapid increase in luciferase transcripts. Similar findings were achieved with UV treatment as well as with cdc25-luc and DHFR-luc reporters and with neuronal PC12 cell cultures (data not shown). Cotreatment with actinomycin D or flavopiridol blocked the increase. Flavopiridol is a CDK inhibitor that protects neurons from camptothecin and other DNA damaging agents and that blocks DNA damage-induced pocket protein hyperphosphorylation and degradation (Park et al., 1997b). These findings thus demonstrate that DNA damage to neurons evokes a rapid, CDK-dependent decrease in repression of E2F-responsive genes.

The E2F-Repressed Genes B-myb and C-myb Are Upregulated in Neurons

To investigate the regulation of endogenous E2F-repressed genes in response to DNA damaging agents, we first carried out semiquantitative RT-PCR on B-myb mRNA isolated from cortical neurons treated for various times with camptothecin. As shown in Figure 2A, under the conditions of our PCR assay, we detected a PCR fragment of the expected length only after 6 and 12 hr of camptothecin treatment, indicating that DNA damage leads to elevated B-myb expression. In contrast, GAPDH-specific primers generated the expected PCR product with equal intensity at all time points of treat-

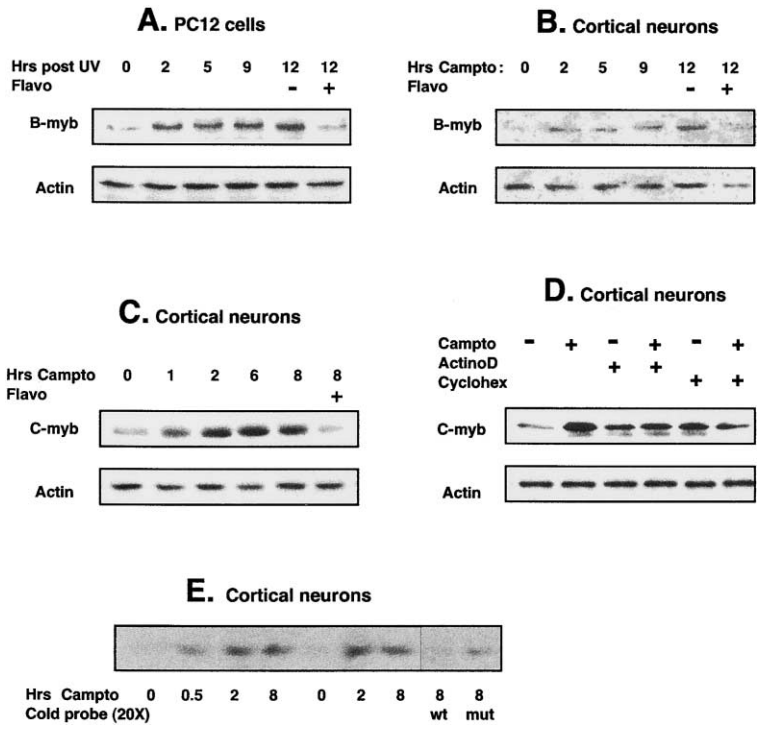

Figure 3. Induction of B-myb and C-myb Proteins in Neuronal PC12 and Cortical Neurons following Exposure to DNA Damaging Agents

(A and B) Induction of B-myb. Cultures were treated as indicated and harvested for total protein. Samples (100 μg protein) were subjected to Western immunoblotting with anti B-myb antiserum. Flavopiridol was added to culture 30 min before UV or camptothecin treatment. Membranes were stripped and reprobed for actin, showing equal loading.

(C and D) Induction of C-myb in cortical neurons. Cultures were treated as indicated, and 60 μg protein per sample was subjected to Western immunoblotting with anti C-myb antiserum and, after stripping, anti-actin antiserum. Flavopiridol, actinomycin D, and cyclohexamide were added 30 min before camptothecin where indicated.

(E) Elevation of Myb DNA binding activity in cortical neurons treated with camptothecin. A ^{32}P-labeled Myb consensus sequence was mixed with extracts of camptothecin-treated cortical neurons for various times as indicated. Electrophoretic mobility shift assays were performed as described in Experimental Procedures. For competition experiments, excess cold probe (20×) was added 10 min before the labeled probe. Reactions were separated by PAGE on 4% nondenaturing gels containing TBE.

ment. For quantitative measurements, we employed real-time PCR analysis using GAPDH mRNA levels for normalization. As shown in Figure 2B, levels of B-myb transcripts doubled within 1–2 hr of camptothecin exposure. Parallel experiments revealed a similar response of C-myb mRNA (Figure 2C). These increases were prevented by actinomycin D (Figures 2B and 2C) as well as by flavopiridol (Figure 2C). The data shown in Figure 2E confirm that both actinomycin and flavopiridol also protected cortical neurons from camptothecin-induced death in these studies.

To examine whether DNA damage also affects the levels of the B-myb and C-myb proteins in neuronal cells, we performed Western blot analyses of whole-cell extracts of UV- and camptothecin-treated PC12 cells and cortical neurons. As shown in Figures 3A and 3B, levels of B-myb protein increased within 2 hr of treatment and remained elevated for at least 12 hr. Similar results were found for levels of C-myb protein in UV- (data not shown) or camptothecin-treated (Figures 3C and 3D) cortical neurons. The increases in Myb proteins were suppressed by flavopiridol (Figures 3A–3C), actinomycin D (Figure 3D), or cyclohexamide (Figure 3D), indicating the elevated protein levels were dependent on both ongoing transcription and translation as well as on CDK activity.

We next determined whether the elevated levels of Myb proteins in camptothecin-treated neurons have the capacity to bind DNA. Figure 3E shows a gel shift carried out using a radiolabeled Myb-specific DNA probe and extracts of camptothecin-treated cortical neurons. Myb binding was barely detectable before treatment and was readily observable within 30 min and persisted to at least 8 hr. Binding was eliminated by excess (20×) unlabeled probe but not by a counterpart with a point mutation in the Myb binding domain.

Taken together, the above findings indicate that the *B-* and *C-myb* genes, whose expression is repressed by E2F, undergo rapid increases in expression in neurons subjected to DNA damage. These observations indicate that DNA damage evokes derepression of E2F-regulated transcripts in neurons.

Expression of E2F Mutant Constructs Promotes Neuronal Apoptosis: Role of Gene Derepression

Although the above observations indicate that DNA damage causes E2F-dependent derepression, they do not exclude an accompanying elevation in E2F-dependent gene transactivation and therefore do not distinguish between the potential roles of these two responses in neuronal death. To achieve this distinction, we transfected a variety of E2F1 constructs (Figure 4A) into neuronal cells and monitored their effects on Bmyb-luc activity and survival.

E2F1(1-368) is a truncated form of E2F1 that lacks both the activation and Rb binding domains (Figure 4A; Sellers et al., 1995). This therefore acts as a dominant negative that suppresses E2F1-dependent transactivation and, by virtue of its inability to bind pocket proteins, displaces endogenous E2F complexes, causing derepression of certain E2F-responsive genes in neurons (Zhang et al., 1999). Figure 4B shows that cotransfection of E2F1(1-368) along with Bmyb-luc resulted in increased expression of the latter by about 4-fold. Similar findings were achieved with cortical and sympathetic neurons (data not shown) as well as with another construct, E2F1(FS409) (Johnson et al., 1993), that contains a frameshift mutation that abolishes the Rb interaction

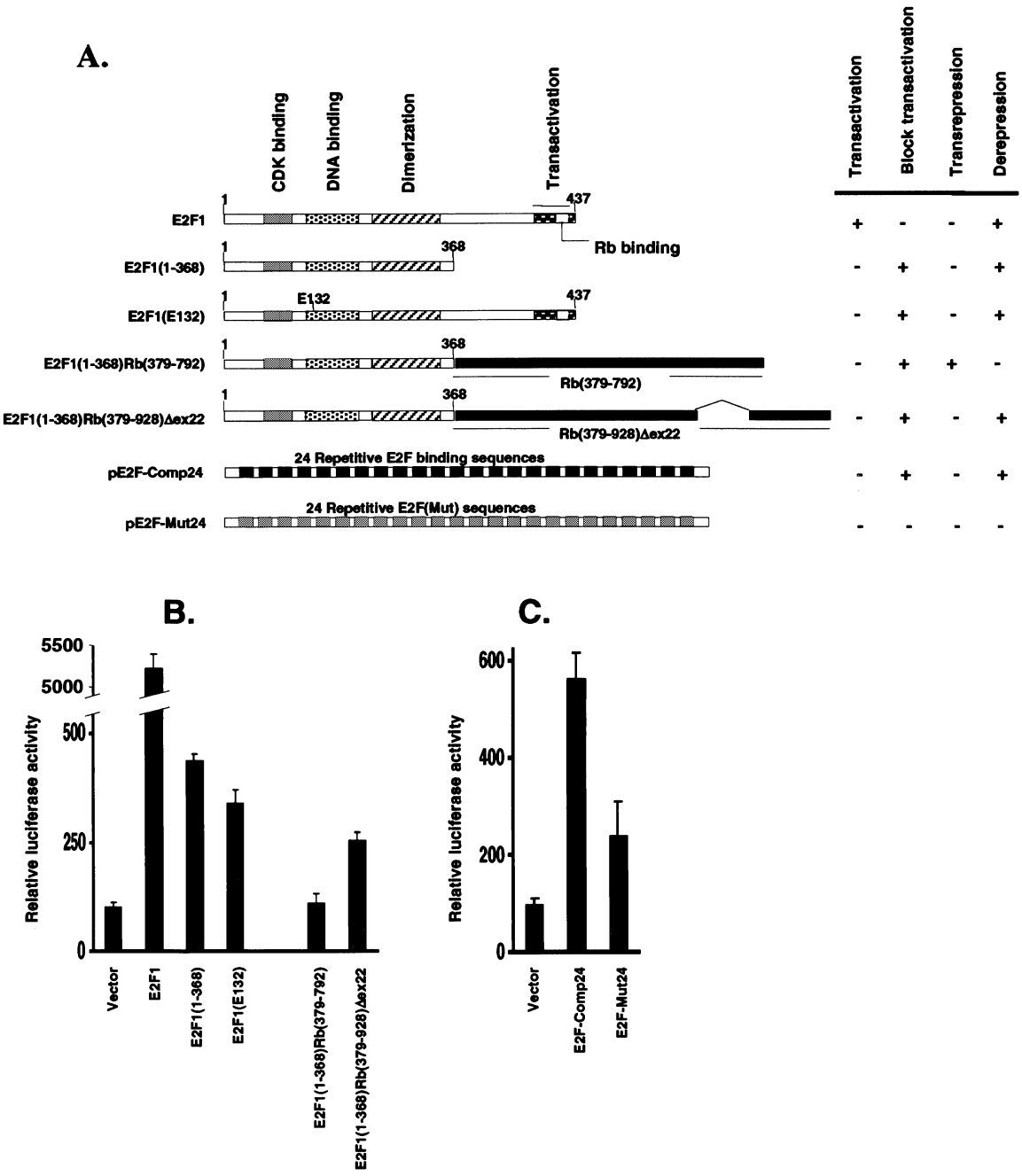

Figure 4. Effect of Different E2F Constructs and E2F Decoys on B-myb Promoter Expression in Neuronal PC12 Cells and in Sympathetic Neurons

(A) Summary of the properties of E2F constructs and E2F decoys used in this study. See text for details.

(B) Upregulation of B-myb promoter expression by E2F activation and E2F derepression in neuronal PC12 cells. The indicated E2F constructs were cotransfected with Bmyb-luc and pcDNA-LacZ. Later (24 hr), cells were harvested and used for luciferase and β-gal assays. Luciferase activity was normalized against β-gal activity. Data represent means ± SEM (n = 3).

(C) An E2F decoy causes derepression of B-myb promoter activity in SCG neurons. SCG neurons were cotransfected with E2F decoys and Bmyb-luc and pcDNA-LacZ as in Experimental Procedures. Relative luciferase activities were obtained as described in (B). Data represent means ± SEM (n = 3).

domain and compromises transactivation activity (data not shown). In contrast, cotransfection with wild-type E2F1 elevated luciferase activity by more than 50-fold (Figure 4B). This difference most likely reflects the capacity of wild-type E2F1 to promote both transactivation and derepression. It is of interest that the degree of regulation of Bmyb-luc reporter activity by E2F1(1-368) is similar in magnitude to that which occurs in response to DNA damage.

We next tested the effect of E2F1 and E2F1(1-368) on

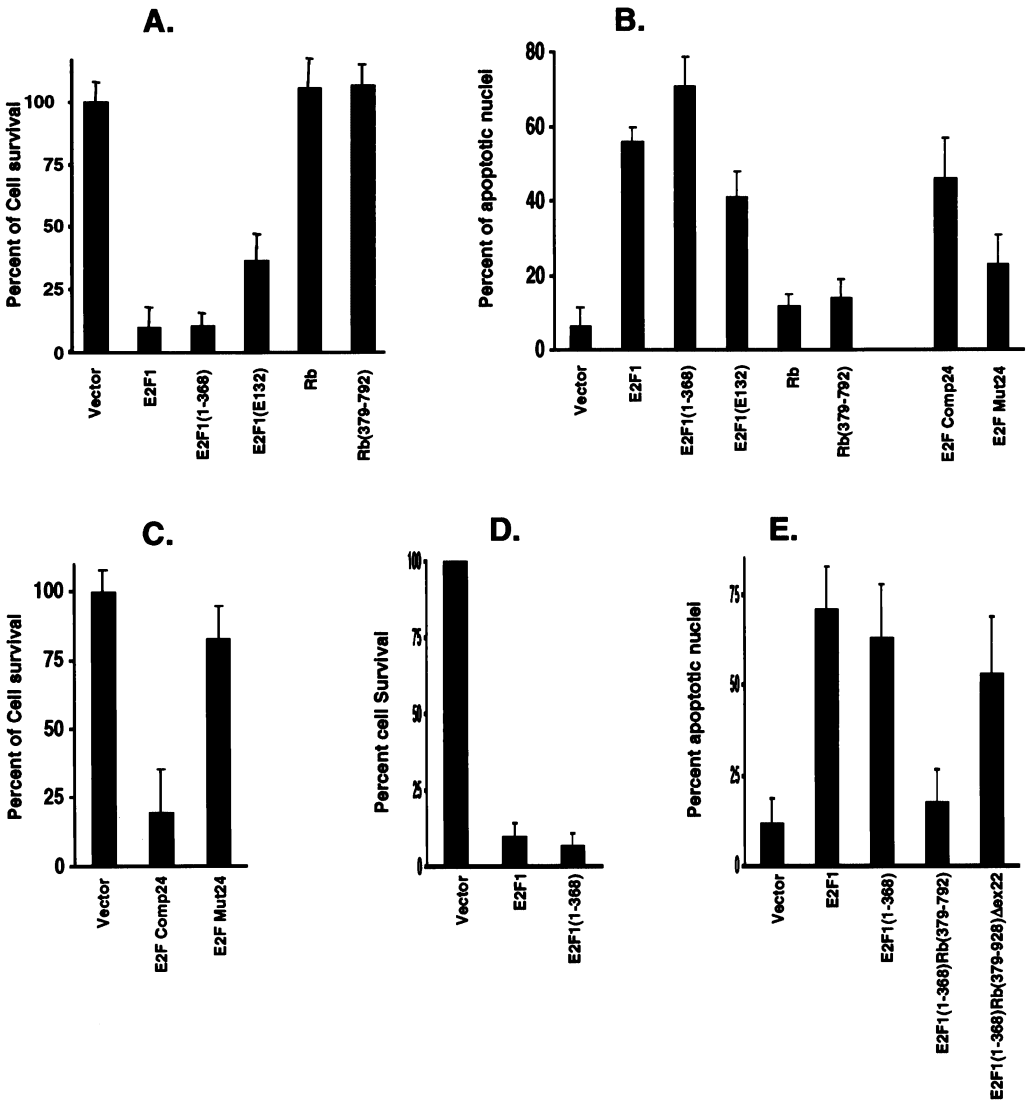

Figure 5. Derepression of E2F-Regulated Genes in Neurons Causes Apoptosis whereas Rb-Promoted Repression of E2F-Regulated Genes Does Not

(A and B) Neuronal PC12 cells. Indicated E2F, Rb, and E2F decoy constructs were cotransfected with pEGFP into neuronal PC12 cells as in Figure 1, and effects were assessed 2 days later. (A) Survival was assessed by strip counting numbers of GFP-positive cells. Results are expressed relative to survival with the control vector alone. (B) Death was assessed by determining the percentage of GFP-positive apoptotic nuclei over the total number of GFP-positive cells scored. Data are presented as means ± SEM (n = 3).

(C–E) Sympathetic neurons. Sympathetic neurons were cotransfected with the indicated E2F decoys (C) and E2F and E2F-Rb fusion constructs (D and E) along with pEGFP. The percentages of surviving neurons (C and D) and of apoptotic nuclei (E) were determined as in (A) and (B).

survival of neuronal PC12 cells (Figures 5A and 5B), sympathetic neurons (Figures 5D and 5E), and cortical neurons (data not shown). In each culture type, in contrast to their huge disparity in upregulating B-myb promoter activity, both constructs were similarly effective in promoting massive death. Similar findings were achieved with E2F1(FS409) (data not shown).

The above findings are consistent with the interpretation that both wild-type and E2F1 mutants induce death by displacing endogenous repressive E2F-pocket protein complexes, thereby relieving repression of genes including those that promote death. Since the E2F mutant constructs lack transactivation activity but possess DNA binding activity that permits displacement of endogenous E2F-repressive complexes, the death response to their expression should not be attributable to E2F-mediated gene activation but rather to E2F derepression. To further test this hypothesis, we employed an additional E2F1 mutant, E2F1(E132), which has mutations that abolish its E2F site binding activity (Johnson et al., 1993). This mutant should not directly displace endogenous E2F1 complexes from DNA but should rather compete for pocket proteins, thereby alleviating the repressive effect of endogenous E2F-pocket protein complexes. As shown in Figure 4B, E2F1(E132) enhances the expression of Bmyb-luc to a degree similar to that achieved with E2F1(1-368). Moreover, this construct was also comparably effective in inducing death of neu-

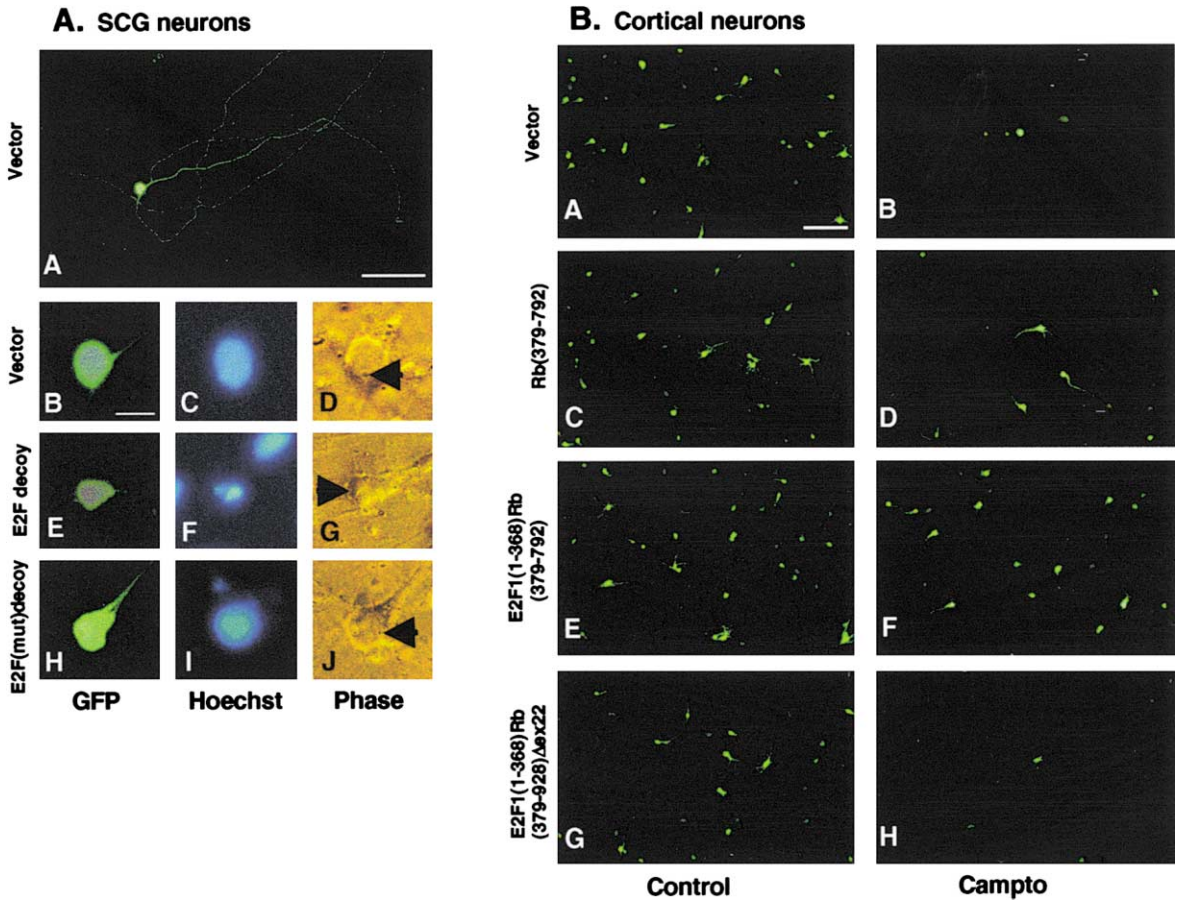

Figure 6. Photographs of Effect of E2F Decoys, Rb(379-792), and E2F-Rb Fusions on Survival of SCG and Cortical Neurons

(A) Expression of an E2F decoy induces apoptosis of SCG neurons. Transfection of the indicated constructs and pEGFP was carried out as in Figure 5. At 2 days after transfection, cells were fixed and stained with Hoechst 33342. Neurons were observed under green fluorescence, UV, and phase. Arrows (panels AD, AG, and AJ) show gold particles associated with Gene Gun transfection. Scale bars represent 100 μm in (AA) and 20 μm in (AB)–(AJ), respectively.

(B) Effect of E2F repression and derepression on survival of cortical neurons. Cortical neurons were cotransfected with pEGFP and the indicated constructs. At 2 days after transfection, half of the transfected cells were exposed to camptothecin (panels BB, BD, BF, and BH). Photos were taken 48 hr later under UV illustration to show GFP-positive cells. Scale bar represents 50 μm.

ronal PC12 cells (Figures 5A and 5B) as well as of sympathetic and cortical neurons (data not shown).

An E2F Binding Site Decoy Causes Transcriptional Derepression and Induces Neuronal Death

As an alternative strategy to E2F1 mutants, we also employed transfection with an E2F binding site "decoy" (E2F-Comp24) consisting of 24 repetitive DNA binding sites for E2F (Figure 4A; He et al., 2000). This construct competes with cellular DNA for endogenous E2F binding activities, thereby suppressing transactivation and also promoting gene derepression (He et al., 2000). Cotransfection of the decoy into sympathetic neurons (Figure 4C) or neuronal PC12 cells (data not shown) along with Bmyb-luc results in a 5-fold elevation of luciferase activity compared with that in cells cotransfected with empty vector. In contrast, a similar construct of E2F binding sites containing a point mutation that suppresses binding of E2F (E2F-Mut24) showed significantly less effect on luciferase activity. Transfection of the decoy construct into sympathetic neurons (Figures 5C and 6) or neuronal PC12 cells (Figure 5B) effectively evoked death; in contrast, the construct containing mutated E2F sites (E2F-Mut24) had relatively little effect on survival. These observations further support the notion that derepression of E2F-repressed genes can evoke neuronal death and that death does not require E2F-dependent gene transactivation.

Repression of E2F-Responsive Genes Rescues Neurons from Apoptosis Induced by DNA Damage

Because E2F derepression occurs in response to neuronal DNA damage and in light of our observations that forced E2F derepression evokes neuronal death, we next wished to determine whether blockade of E2F derepression would suppress death of neurons. One strategy to this end was to force expression of wild-type Rb or of phosphorylation-resistant Rb small pocket [Rb(379-795); Chow and Dean, 1996]. A different phosphorylation-resistant Rb mutant was previously found to partially protect cortical neurons from death evoked by

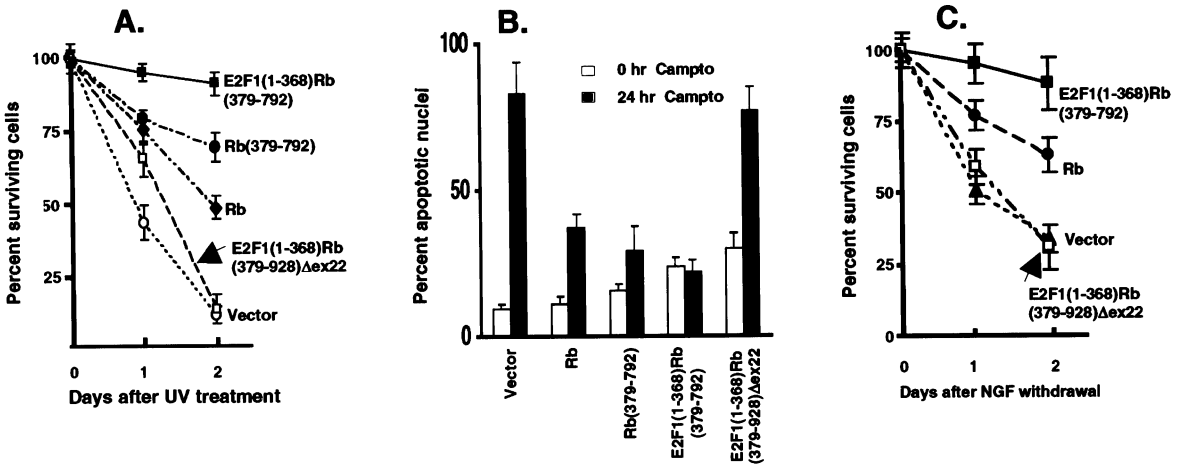

Figure 7. Rb- and E2F-Rb-Mediated Gene Repression Protects Neuronal PC12 Cells from Apoptosis Induced by DNA Damaging Agents and Sympathetic Neurons from NGF Withdrawal

(A) Neuronal PC12 cells were cotransfected with indicated constructs and GFP and 2 days later were exposed to UV irradiation (650 J/m^2). GFP-positive cells were quantified on the following 2 days by strip counting. Data are expressed as relative to day 0 (before irradiation) and are given as means ± SEM (n = 3).

(B) Neuronal PC12 cells were transfected with the indicated constructs and 2 days later were treated with camptothecin. The percentages of apoptotic nuclei were assessed before and 24 hr after camptothecin treatment as described in Experimental Procedures. Data are expressed as relative to untreated controls and are given as means ± SEM (n = 3).

(C) Sympathetic neurons were cotransfected with the indicated constructs and GFP and 2 days later were deprived of NGF. Numbers of GFP-positive cells were evaluated at each indicated time, and data are expressed relative to day 0 (before NGF withdrawal) and are given as means ± SEM (n = 3).

DNA damage (Park et al., 2000). These constructs had minimal effect on survival of neuronal PC12 cells in the absence of DNA damage (Figures 5A and 5B) and significantly depressed death evoked by UV (Figure 7A) or camptothecin (Figure 7B) treatment. Similar results were achieved with sympathetic (data not shown) and cortical (Figure 6 and data not shown) neurons. The better protection conferred by Rb(379-795) as compared to wild-type Rb at 2 days following UV irradiation is consistent with the notion that phosphorylation of Rb is an important step in its inactivation and eventual removal during the apoptotic process.

Although the above strategy reinforces the importance of non- or hypophosphorylated pocket proteins in neuronal survival, they do not distinguish between potential actions of Rb in blocking E2F-dependent transactivation or in facilitating E2F-dependent active gene repression. We therefore tested a construct encoding a fusion protein [E2F1(1-368)-Rb(379-792) that includes the DNA binding and dimerization domains of E2F1 and the pocket domain of Rb; Figure 4A; Sellers et al., 1995]. This fusion protein not only should be unable to activate E2F-responsive genes, but should repress them by means of the Rb moiety. As a control, we used the construct E2F1(1-368)-Rb(379-928)Δex22, which has an exon 22 deletion and impaired function for repression (Sellers et al., 1995; Figure 4A). When tested for derepression activity by cotransfection with Bmyb-luc, E2F1(1-368)-Rb(379-792) exerted little effect (Figure 4B), indicating that E2F in neuronal cells is repressed to such a level that expression of another E2F repressor does not further affect reporter activity. In contrast, E2F1(1-368)-Rb(379-928)Δex22 coexpression increased reporter activity by 2.5-fold (Figure 4B), indicating that this impaired repressor displaces endogenous E2F-pocket protein complexes, thereby inducing derepression. Similar observations were made with sympathetic and cortical neurons (data not shown). When the two constructs were tested for effects on survival of sympathetic neurons, E2F1(1-368)-Rb(379-792) had little if any effect, whereas E2F1(1-368)-Rb(379-928)Δex22 induced a significant level of death (Figures 5E and 6B). These observations are consistent with and support our observations that derepression of E2F-responsive genes is sufficient to induce apoptosis and that active repression of E2F-responsive genes rather than blockade of E2F activation is required for neuron survival.

We next evaluated the role of E2F-mediated gene derepression in death evoked by DNA damage. Neuronal cultures were transfected with the two E2F-Rb fusion constructs, subjected to DNA damage, and then assessed for survival. As shown in Figure 7, E2F1(1-368)-Rb(379-792) but not E2F1(1-368)-Rb(379-928)Δex22 provided strong protection from UV irradiation (Figure 7A and data not shown for sympathetic and cortical neurons) and camptothecin (Figures 6B and 7B). These findings further support the conclusion that derepression of E2F-responsive genes is required for neuronal apoptosis and that repression of such genes is protective against neuronal apoptosis induced by DNA damage.

Forced Expression of B-myb and C-myb Induces Neuronal Death

If derepression of E2F-responsive genes plays a role in the neuronal apoptotic response, then forced expression of at least some of these might be anticipated to induce death. To test this, we transfected neuronal PC12

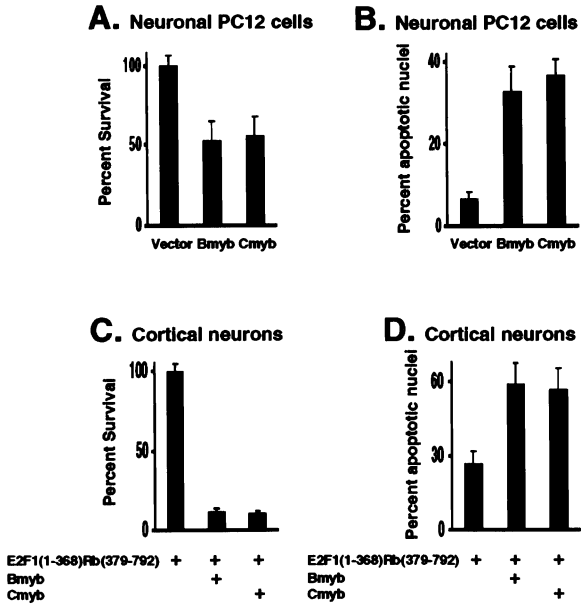

Figure 8. Overexpression of B-myb and C-myb Induces Apoptosis of Neuronal PC12 Cells and Cortical Neurons

(A and B) B-myb and C-myb cause apoptosis of neuronal PC12 cells. B-myb and C-myb were cotransfected with pEGFP, and survival was assessed both as percentage of surviving GFP-positive cells (A) and as percentage of apoptotic nuclei (B). Data are given as means ± SEM (n = 3).

(C and D) B-myb and C-myb cause apoptosis in cortical neurons even when derepression of E2F-responsive genes is blocked. Neurons were cotransfected with pEGFP and the indicated constructs. Survival was accessed as in (A) and (B). Data are given as means ± SEM (n = 3).

cells and cortical neurons with B-myb and C-myb and 48 hr later monitored both numbers of surviving cells and cells with apoptotic nuclei. As shown in Figures 8A and 8B, both constructs evoked death. According to our model, these genes should act downstream of E2F derepression and thus should induce death even when E2F-mediated repression is enforced. The data in Figures 8C and 8D indicate that, in contrast to its capacity to inhibit DNA damage-induced death, E2F-specific transrepressor E2F1(1-368)-Rb(379-792) does not inhibit death evoked by either B-myb or C-myb.

E2F-Mediated Derepression Is Involved in Neuronal Death Induced by NGF Withdrawal

As in the case of DNA damage, death of sympathetic neurons and neuronal PC12 cells evoked by NGF withdrawal is suppressed by flavopiridol, a CDK4/6 inhibitor (Park et al., 1996a). Likewise, CKIs and dominant-negative CDK4 and 6 (but not CDK2) also protect such cells from NGF deprivation (Park et al., 1997b), and, in this model, elevation of C-myb mRNA has been reported (Estus et al., 1994). To test whether E2F-mediated derepression is also a component of death in this paradigm, we transfected the Bmyb-luc reporter into neuronal PC12 cells and followed luciferase activity during the course of NGF withdrawal. Reporter activity was more than doubled within 2 hr of NGF withdrawal and was elevated by 6-fold within 10 hr (Figure 1F), indicating that NGF withdrawal relieves repression of the B-myb promoter by E2F. In support of the latter, this upregulation was blocked by the presence of flavopiridol (Figure 1F). Examination of the endogenous levels of C-myb transcripts confirmed that these undergo a similar elevation following NGF withdrawal and revealed that flavopiridol eliminated this response (Figure 2D). We also observed an increase in levels of endogenous B-myb protein within 2 hr of NGF withdrawal (data not shown). Thus, derepression of the Myb promoters is induced by NGF withdrawal as well as by DNA damaging agents. We next tested whether blockade of E2F derepression by the E2F-Rb fusion protein would protect neurons from apoptosis induced by NGF withdrawal (Figure 7C). We cotransfected sympathetic neurons with GFP and different Rb constructs, and, 2 days later, NGF was removed and the medium replaced with one containing anti-NGF antibody. Counts of GFP-positive neurons revealed that E2F1(1-368)-Rb(379-792) promoted survival rates of 95% and 88% after 1 and 2 days of NGF withdrawal, respectively. In contrast, the corresponding mutant version of the fusion protein, E2F1(1-368)-Rb(379-928)Δex22, showed no protection compared with the vector control (ca. 60% and 35% survival after 1 and 2 days, respectively). These results indicate that E2F derepression is an underlying mechanism of apoptosis evoked by NGF withdrawal as well as by DNA damage.

Discussion

Role of E2F-Dependent Gene Repression and Derepression in Neuronal Survival and Death

Prior studies have implicated the cyclin D-CDK4/6-pocket protein-E2F pathway in a variety of neuronal death paradigms and have suggested that E2F is a key regulator of at least some of the transcriptional events that are required for neuronal apoptosis (Park et al., 1996a, 1997a, 1997b, 1998, 2000; Liu and Greene, 2001). Because E2F may function either as a transcriptional activator or transcriptional corepressor, we asked which of these two mechanisms might be important for neuronal survival and for neuronal death.

Our findings revealed strong repression of E2F reporter constructs when these were expressed in neuronal PC12 cells and neurons. This indicates that E2F functions primarily as a transcriptional repressor in such cells. Presumably, the absence of E2F transactivational activity and the presence of E2F-dependent repression play an important role in maintaining neurons and NGF-treated PC12 cells in a postmitotic state. This notion is supported by our observation that the activity of an E2F reporter was substantially higher when expressed in proliferating PC12 cells.

A second major observation was that forced derepression of E2F-responsive genes caused neuronal cell death, even in the presence of a trophic factor such as NGF. This was achieved by two different approaches, namely, overexpression of E2F dominant-negative proteins lacking activation domains or with mutant DNA binding domains and by transfection with an E2F DNA decoy with multiple E2F binding sites. Such findings indicate that E2F-mediated gene repression not only occurs in neurons, but is required for their survival.

A third finding was that DNA damaging agents or NGF withdrawal led to derepression of E2F-inhibited genes. This was indicated by elevated expression of E2F reporter constructs as well as by transcription-dependent elevation of endogenous B- and C-myb mRNAs and proteins. E2F acts as a corepressor for these genes in healthy neurons, and, hence, the increase in their expression following DNA damage or NGF withdrawal supports the occurrence of derepression during the death process.

A fourth observation was that death promoted by DNA damage or NGF withdrawal was blocked when derepression of E2F-responsive genes was abolished. This was achieved by transfection with a construct encoding an E2F-Rb fusion protein that should act as a transcriptional repressor, even under conditions in which cyclin D-CDK4/6 activity is elevated.

Regulation of E2F-Mediated Derepression

Recent studies suggest a sequential role for E2F-dependent derepression and activation in cell cycle progression (Harbour and Dean, 2000; Harbour et al., 1999). In nonproliferating cells, association of pocket proteins with E2F inhibits its transactivational activity and actively promotes gene repression (Zhang et al., 1999). This has the net effect of silencing genes associated with DNA synthesis and mitosis and contributes to the quiescent state. During the transition through G1 toward S, cyclin D-CDK4/6 activity becomes elevated, leading to an initial phosphorylation of pocket proteins. The effect of this phosphorylation is to relieve repression by E2F-pocket protein complexes, presumably by affecting association with HDAC and CtBP (Harbour and Dean, 2000; Meloni et al., 1999). This derepression evokes induction of a subset of genes that may include many associated with cell cycle progression, such as B- and C-myb, cdc6, and cyclin E. The elevation of cyclin E is postulated to activate CDK2 and permits E2F transactivational activity by further phosphorylation of pocket proteins. This in turn induces additional genes required for subsequent steps in cell cycle progression (Harbour and Dean, 2000).

Both the present and past observations are consistent with the interpretation that apoptotic stimuli, including DNA damage and NGF deprivation, lead to events that favor E2F-promoted gene derepression and that this, rather than E2F-dependent gene activation, plays a required role in neuronal apoptosis. For instance, neuronal death is blocked by G1/S inhibitors but not by those acting at later stages in the cell cycle (Farinelli and Greene, 1996). This suggests a particular vulnerability at the G1/S checkpoint, which is at the place in the cycle at which E2F-mediated derepression appears to occur (Harbour et al., 1999). In addition, dominant-negative CDK4 and CDK6 protect neurons from apoptosis evoked by DNA damaging agents and NGF withdrawal, whereas dominant-negative CDK2 does not (Park et al., 1997a, 1998). In light of the postulated specific roles for CDK4/6 and CDK2 discussed above, these observations again favor gene derepression rather than activation as a required player in the death process. Several studies have reported that overexpression of E2F promotes death of postmitotic neurons (Athanasiou et al., 1998; Hou et al., 2000). Although the favored interpretation was that E2F stimulated transactivation of death genes, an equally and perhaps more plausible interpretation would be that excess exogenous E2F displaced endogenous repressive E2F-pocket protein complexes at E2F sites, thereby causing derepression of apoptotic genes. This interpretation is supported by our observations that E2F mutants without a transactivation domain and an E2F decoy construct also promote neuronal death. Finally, overexpression of phosphorylation-resistant Rb mutants partially protected neurons from death evoked by DNA damage (Park et al., 2000; present study) or NGF deprivation (present study). Here again, our interpretation is that the Rb mutants replaced phosphorylated endogenous pocket proteins in complexes with E2F, thereby retaining gene repression. This interpretation is strongly supported by our observations that E2F-tethered Rb effectively protected neurons from death evoked by either DNA damage or NGF withdrawal.

Although our data are consistent with the required involvement of E2F-mediated gene derepression in neuronal death, they do not support an obligatory role for E2F-dependent gene transactivation in this process. If the latter possibility were the case, then dominant-negative E2F constructs and the E2F binding decoy should have promoted survival rather than death. Of additional potential relevance, past studies in several nonneuronal cell lines have shown that, while E2F activation promotes cell proliferation, it is not required to promote apoptosis (Hsieh et al., 1997; Phillips et al., 1997).

A Pathway to Neuronal Death Evoked by DNA Damage or NGF Withdrawal

Taken together, our findings suggest a pathway by which DNA damage or NGF withdrawal leads to E2F-dependent gene derepression and to neuronal death (Figure 9). The first well-defined step in this pathway is elevation of cyclin D-associated kinase activity. Past evidence indicates that such an increase is driven by a combination of events, including elevation of cyclin D-associated kinase activity (Park et al., 1998) as well as by translocation of cyclin D to the nucleus (Padmanabhan et al., 1999). The enhancement of cyclin D-associated kinase activity promotes hyperphosphorylation of pocket proteins bound to E2F, which compromises their capacity to mediate gene repression. The consequent deregulation of genes elevates cellular levels of proapoptotic proteins that contribute to neuronal death.

Although the E2F derepression pathway described here may be required and sufficient for neuronal death, this does not exclude involvement of other pathways in this regard. For instance, activation of the JNK/c-Jun pathway also appears to be required for neuronal death evoked by DNA damage and NGF deprivation as well as additional causes (Maroney et al., 1999). However, activation of the two pathways appears to be independent (Park et al., 1996b). In addition, p53 also appears to play a required role in neuronal death elicited by DNA damage (cf. Morris et al., 2001) and NGF deprivation (Aloyz et al., 1998). In this respect, it had been shown in proliferation-competent cells that E2F1 may induce p53-dependent (Qin et al., 1994) as well as p53-independent apoptosis (Macleod et al., 1996). Very recent evi-

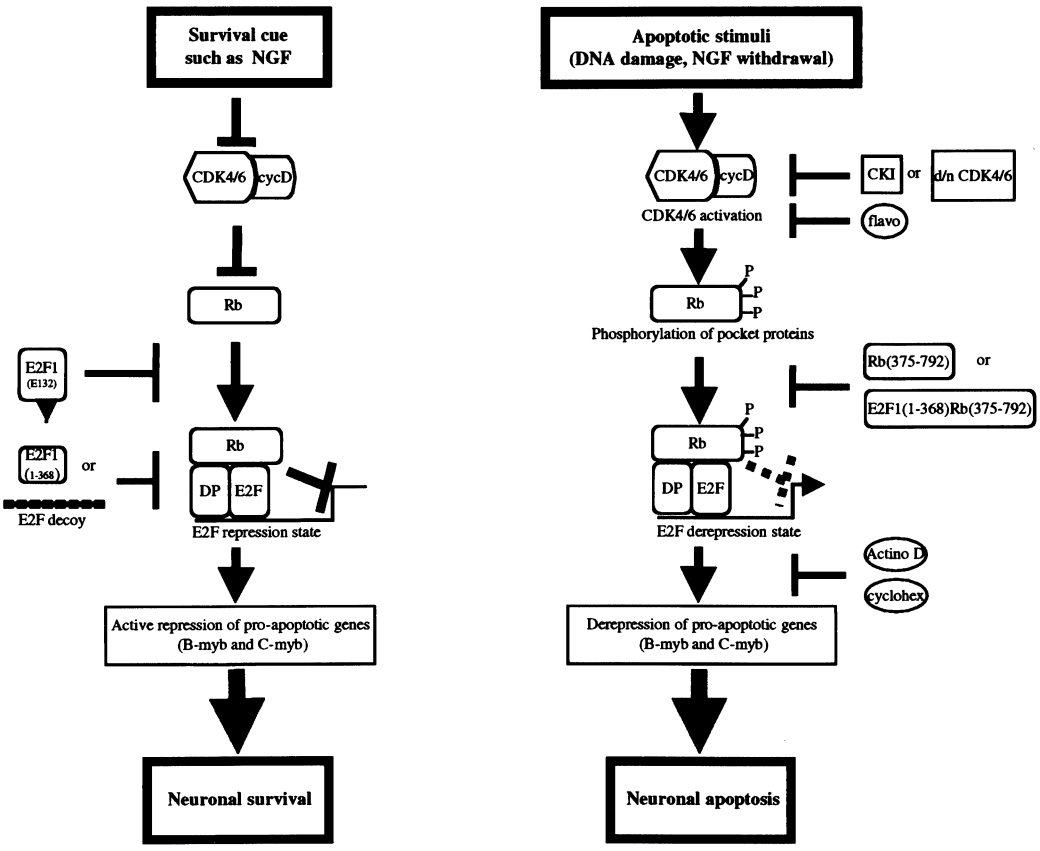

Figure 9. A Model for E2F Repression/Derepression in Neuronal Survival and Apoptosis

dence (Morris et al., 2001), however, indicates that the E2F and p53 pathways in neurons are independently regulated in response to DNA damage; in the case of NGF deprivation, E2F activation has been suggested to be downstream of the JNK/c-Jun pathway (Aloyz et al., 1998). In proliferation-competent cells, overexpression of E2F can also cause apoptosis by direct induction of the p53 family member p73 (Stiewe and Putzer, 2000). However, at least for NGF deprivation, death appears to be associated with disappearance of the p73 derivative p63 rather than to upregulation of p73 itself (Pozniak et al., 2000). Thus, as previously suggested (Park et al., 1996b), neuronal death appears to result from the activation and convergence of several independent transcriptional pathways.

Myb Proteins and Neuronal Death

Although genes that are subject to E2F-mediated derepression seem to play a prominent role in neuronal apoptosis in response to DNA damage and NGF withdrawal, the identities of these proapoptotic genes have yet to be fully defined. However, among the candidates are *B-* and *C-myb*. A relatively low, basal level of these proteins was detected in control neuronal cultures. It is unclear whether this reflects a basal level of death or that Myb proteins play physiological functions in healthy neurons. Of relevance to this, in situ hybridization is reported to reveal low levels of C-myb mRNA in many different neuron types in adult brain (Shin et al., 2001).

Our experiments revealed rapid induction of the B- and C-myb proteins in cortical neurons, sympathetic neurons, and neuronal PC12 cells in response to DNA damage or NGF withdrawal and that forced expression of these proteins causes neuronal apoptosis. Transfection with Myb family members has been shown to promote cell cycle progression and to cause death of non-neuronal cell types as well (Athanasiou et al., 1996; Bies and Wolff, 1995; Sala et al., 1996). Such observations support the possibility that elevation of Myb protein levels in postmitotic neurons contributes to death evoked by DNA damage or NGF withdrawal. Our finding that Myb proteins promoted death even when cotransfected with an E2F-Rb fusion construct indicates that these act even when E2F-dependent derepression is blocked and therefore can be sufficient to induce death in the absence of other derepressed genes. The mechanism by which Mybs induce neuronal death is unclear, but, given that these proteins are transcription factors, it seems likely that they in turn regulate proapoptotic genes.

E2F-Mediated Derepression in Other Paradigms of Neuronal Death

The data presented here pertained specifically to neuronal death promoted by DNA damaging agents and NGF deprivation. However, similar mechanisms may apply to neuronal death evoked by a variety of additional apoptotic stimuli. Death evoked by application of β-amyloid

protein (Giovanni et al., 1999) and oxygen deprivation (Osuga et al., 2000) as well as by DNA damage (Park et al., 1996b) and NGF deprivation (Park et al., 1996a) is suppressed by flavopiridol, a CDK4/6 inhibitor. Furthermore, hyperphosphorylation and cleavage of pocket proteins is associated with repolarization-induced death of cerebellar granule cells (Padmanabhan et al., 1999). Thus, E2F-mediated derepression may well be a component of death in a variety of neuronal death paradigms.

Experimental Procedures

Culture and Transfection of PC12 Cells, Cortical Neurons, and Sympathetic Neurons

PC12 cells, embryonic rat cortical neurons, and neonatal rat superior cervical ganglion sympathetic neurons were cultured as previously described (Greene and Tischler, 1976; Park et al., 1998).

Transfection protocols were as follows.

PC12 Cells

Naive or neuronal PC12 cells in 24-well dishes were transfected using LipofectAMINE plus or LipofectAMINE 2000 (Life Technologies) following the manufacturer's standard protocol. All cDNA constructs were used at 1 μg/well; E2F decoys were at 0.5 μg/well.

Cortical Neurons

Transfection of cortical neurons was carried out 3 days after plating as described for PC12 cells, except that the transfection medium was replaced with fresh medium after 5–6 hr.

Sympathetic Neurons

On the third day after plating, neurons were transfected with a Helios Gene Gun System (BioRad). In brief, DNA was loaded on microcarriers (1.6 μm) with a ratio of DNA:gold carrier (2 μg:1 mg) according to manufacturer's protocol (BioRad). Medium was carefully removed from the wells shortly before shooting and replaced immediately after. The Gene Gun was held perpendicularly over the culture dish and about 1.5 cm above the surface of the cell layer. Pressure was set at 200 psi. Even distribution of gold particles was monitored by microscopic observations.

UV Irradiation, Camptothecin Treatment, and NGF Withdrawal

Neurons or PC12 cells were exposed to UV irradiation at 350 J/m^2 (cortical and sympathetic neurons) or 650 J/m^2 (PC12 cells), respectively, in a Stratolinker UV crosslinker (Stratagene) (Park et al., 1998). For camptothecin (Sigma) treatment, stock (10 mM in DMSO) was diluted and dissolved in medium and then added to cultures to a final concentration of 10 μM. Flavopiridol (L86-8275, [(-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-benzopyran4-1], a generous gift from Dr. Peter J. Worland, National Cancer Institute), actinomycin D, and cyclohexamide (both from Sigma) were added 30 min before UV or camptothecin treatment as indicated.

For NGF withdrawal, sympathetic neurons or PC12 cell cultures were washed twice with NGF-free RPMI 1640 medium and treated with anti-hNGF antiserum (Sigma, 1:200 dilution).

Survival Assay

Strip Counting

In brief, a 24-well plate was placed onto a moveable microscope stage. The numbers of GFP-positive cells in a defined strip across the culture were assessed by phase and fluorescence microscopy. The same strip was scored each day during the time course. The percentage of surviving cells was calculated relative to the number present in the same well before treatment.

Apoptotic Nuclei

At various time points, neurons or PC12 cells were fixed in 4% formaldehyde for 10 min and then, after washing with PBS, exposed to Hoechst 33342 (Sigma) at 1 μg/ml in PBS and 0.1% Triton X-100 for 15 min. Intact, GFP-positive neurons with intact nuclei were scored as viable, whereas those with condensed nuclei and fragmented chromatin were counted as dying or dead. Apoptotic rates were represented as the percentage of apoptotic cells out of the total number of GFP-positive cells assessed. All experiments were performed in triplicate and are reported as mean ± SEM (n = 3).

Luciferase Assay

Neurons or PC12 cells were transfected as described above, with appropriate luciferase reporter and pcDNA-LacZ with or without other DNAs. At 24 or 48 hr later, cells were washed with cold PBS, triturated off the plates, and pelleted in microcentrifuge tubes. Cell pellets were lysed in buffer provided in the Promega Luciferase System (Promega). Luciferase assay was carried out according to the manufacturer's instructions, using a TD-20/20 Luminometer (Turner Designs). Relative luciferase activities were obtained by normalizing the luciferase activity against β-gal activity. Data are presented in the text and figures as mean ± SEM (n = 3).

Semiquantitative RT-PCR and Quantitative PCR

RNA was isolated from cells using TriReagent (Molecular Research Center) as described by the manufacturer. Reverse transcription was carried out using Superscript II reverse transcriptase (Life Technologies) following the manufacturer's instructions. Semiquantitative PCR (GeneAmp PCR System 9700, Perkin-Elmer) was carried out using Platinum Taq DNA polymerase (Life Technologies) following the manufacturer's instructions. For B-myb, oligonucleotides GGGCTCAGGCATTGGCACACC and GTGTGGGGAGTGTTGTCC ATGG were used (27 to 29 cycles). For GAPDH, oligonucleotides ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA were used (20 to 21 cycles).

Quantitative PCR was carried out using a Cepheid SmartCycler following the manufacturer's specifications. cDNA was added to a 25 μl volume reaction mix containing Ready-to-go Beads (Amersham Manheim Pharmaceuticals) and SYBR Green I (Molecular Probes) together with appropriate primers at 0.2 μM each. Analyses of growth curves of real-time fluorescence and of melting curves were carried out as described before (Troy et al., 2001). GAPDH was used to normalize input cDNA. Levels of luciferase mRNA in reporter transfection assays were also normalized by β-gal mRNA levels. The same oligonucleotides listed above were used for B-myb and GAPDH: CGGTCCCCTGAAGATGCTACCT and GGTCCTCTG TCTTCCCACAGGA for C-myb; CGCTGCTGGTGCCAACCCT and GGCGTTGGTCGCTTCCGGA for luciferase; CCGACCCAGCGCCCG TTGCA and GGCCCGCACCGATCGCCCTT for β-gal.

Western Blot Analyses

Cell extracts from cortical neurons or neuronal PC12 cells were prepared as described (Padmanabhan et al., 1999). Protein concentration was determined by Bradford assay (BioRad). Western immunoblotting was carried out as described (Park et al., 1998). Anti B-myb antibody (Santa Cruz) and anti C-myb antibodies (Santa Cruz and Upstate Laboratories) were used at 1:200, 1:200, and 1:1000, respectively. Prior to reprobing with anti-actin (1:500), membranes were exposed overnight at room temperature to stripping buffer (62.5 mM Tris [pH 6.8], 100 mM BME, 2% SDS) and washed extensively with PBS.

Gel Shift Assay

Electrophoretic mobility shift assays (EMSAs) were performed essentially as described by Chellappan et al. (1991). Where appropriate, a 20-fold excess of unlabeled probe (wild-type or with a point mutation; Santa Cruz) was added to the reactions 10 min before the ^{32}P-labeled Myb probe (Santa Cruz) was added.

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