B-Myb and C-Myb Play Required Roles in Neuronal Apoptosis Evoked by Nerve Growth Factor Deprivation and DNA Damage

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Activation of cell cycle elements plays a required role in neuronal apoptosis associated with both development and neurodegenerative disorders. We demonstrated previously that neuron survival requires gene repression mediated by the cell cycle transcription factor E2F (E2 promoter binding factor) and that apoptotic stimuli lead to de-repression of E2F-regulated genes and consequent death. However, the downstream mediators of such death have been unclear. The transcription factors B- and C-myb are E2F-regulated genes that are induced in neurons by apoptotic stimuli. Here, we examine the role of B- and C-myb in neuron death. Antisense and siRNA constructs that effectively block the upregulation of B- and C-myb provide substantial protection against death of cultured neuronal PC12 cells, sympathetic neurons, and cortical neurons elicited by either NGF withdrawal or DNA damage. There is also significant protection from death induced by direct E2F-dependent gene de-repression. Our findings thus establish required roles for B- and C-myb in neuronal apoptosis.

Key words: Myb; E2F; NGF; neuron; apoptosis; cell cycle

Introduction
Neuronal apoptosis plays an indispensable role in neurogenesis and is an underlying cause for neurodegeneration. Recent findings suggest that apoptosis of postmitotic neurons is often associated with activation of the cell cycle machinery (Smith et al., 2000; Copani et al., 2001; Liu and Greene, 2001b; Becker and Bonni, 2004; Greene et al., 2004). Abundant evidence has been presented that supports the following model for involvement of cell cycle molecules in neuron death (Park et al., 1998a,b; Liu and Greene, 2001b; Greene et al., 2004). In viable neurons, the cell-cycle-associated transcription factor E2F (E2 promoter binding factor) is silenced by association with Rb family members and forms complexes that repress death-promoting genes. Apoptotic stimuli such as neurotrophic factor deprivation and DNA-damaging agents lead to activation of Cdk-4 and/or -6 (cyclin-dependent kinases), which in turn phosphorylate members of the Rb family. The latter phosphorylation event causes dissociation of E2F-Rb family repressor complexes and consequent de-repression of genes that lead to neuron death.

Genes with known susceptibility to E2F-dependent repression include the Myb transcription factor family members B-myb and C-myb (Lam and Watson, 1993; Zwicker et al., 1996). Although B- and C-myb play important roles in cell cycle progression (Oh and Reddy, 1999), they have also been associated with proapoptotic activity (Selvakumaran et al., 1994; Bies and Wolff, 1995; Sala et al., 1996; Tashiro et al., 2004). With respect to nerve cells, C-myb transcripts are induced in sympathetic neurons by NGF withdrawal or exposure to a DNA-damaging agent (Estus et al., 1994; Besirli et al., 2003). Furthermore, we found that B- and C-myb proteins are upregulated in neurons exposed to apoptotic stimuli and that this occurs via E2F-dependent gene de-repression (Liu and Greene, 2001a). We further observed that overexpression of B- or C-myb is sufficient to induce death of cultured cortical neurons, sympathetic neurons, and neuronal (i.e., NGF-treated) PC12 cells. In light of these findings, we have investigated here whether B- and C-myb play required roles in neuron death.

Materials and Methods
Materials. Platinum TaqDNA polymerase and LipofectAMINE 2000 were from Invitrogen (San Diego, CA). Anti-human NGF and anti-GFP (green fluorescent protein) antisera, camptothecin, and Hoechst 33342 were from Sigma (St. Louis, MO). Anti-B-myb and anti-C-myb antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant NGF was a kind gift from Genentech (San Francisco, CA).

Cell culture. PC12 cells were cultured as described previously (Greene and Tischler, 1976). Neuronal PC12 cells were generated by exposure to NGF (100 ng/ml) in RPMI 1640 medium with 1% horse serum for 7 d. Embryonic rat cortical neurons and neonatal rat superior cervical ganglion (SCG) sympathetic neurons were cultured as described previously.
(Park et al., 1998b). For NGF deprivation, cell cultures were washed with NGF-free medium twice, and anti-NGF antibody (1:100) was added. Control cells were washed with serum-free medium and maintained in medium supplied with NGF without serum. HEK293 cells were cultured in DMEM with 10% fetal bovine serum.

**Plasmids.** Constructs and their kind sources were as follows: antisense B-myb (A. Sala, Institute of Child Health, London, UK and B. Calabretta, Thomas Jefferson University, Philadelphia, PA), antisense C-myb (G. Siu, Columbia University, New York, NY), E2F1(1–368) (W. Sellers, Harvard University, Boston, MA), and E2F1(1–374) (K. Helins, European Institute of Oncology, Milan, Italy). B-myb and C-myb siRNAs were prepared in the pU6 vector using the GeneSupressor System according to the instructions of the manufacturer (Imagenex, Sorrento Valley, VA) based on the sequences 5’- AAGTCATTGAGCTGTCAGAAGTATGGCAC and 5’-GAAGACCAAAAGGTCATTGAGCTGGTCAAGAAGTATGGCAC and 5’ GTGCCATACCTCTTGACCAGCTACTGCAATGACTTGTTCCT-C for B-myb and primers 5’-TATGAGTTCTCCCTAAACAGTTCCAGCACCCTGGAACCTGGTG and 5’-CAGGCGCAGGTTTTCATGTTTGGCTGAGGTAAAGAAGATCTTATA for C-myb. Sequences of all genes generated from PCR were verified to be correct.

**Transfections.** DNA was prepared with Plasmid Maxi kits (Qiagen, Hilden, Germany). PC12 cells, cortical neurons, and SCG neurons were cotransfected with 0.4 µg of pCMS-EGFP, 0.2 µg of antisense or siRNA construct against B-myb or C-myb, and 0.4 µg of plasmid filler (pDNA) or plasmid expressing E2F1(1–374) or E2F1(1–368) (when these constructs were used) per well in 24-well dishes using 1 µl of LipofectAMINE 2000. For Western blotting analysis, cycling PC12 cells were cotransfected with 1 µg of pCMS-EGFP and 1 µg of control vector or antisense or siRNA construct against B-myb or C-myb per well in six-well dishes using 2 µl of LipofectAMINE 2000. Transfection medium was maintained for 6 hr and was then replaced with fresh complete medium. Camptothecin treatment (10 µM), NGF withdrawal, and survival assays were initiated 24 hr after transfection (day 0). To verify that the transfected cells in cortical cultures were indeed neurons, we stained transfected cultures in parallel experiments with monoclonal antibodies against neuron-specific tubulin (TuJ1), nestin, and GFAP. Among the transfected cells in cortical cultures were indeed neurons, we stained transfected cultures in parallel experiments with monoclonal antibodies against neuron-specific tubulin (TuJ1), nestin, and GFAP. Among the transfected GFP^+^ cells, 97% (349 of 360) were TuJ1^+^, whereas <1% were either nestin^+^ or GFAP^+^. HEK293 cells were transfected as described previously (Liu and Greene, 2001a).

**Immunostaining.** Immunostaining was performed as described by Angelastro et al. (2003). Briefly, cortical cultures were fixed with 4% paraformaldehyde–2% sucrose in PBS for 15 min. After three washes in PBS, the cells were blocked in 10% non-immune goat serum and 0.3% Triton X-100 for 1 hr. The cultures were immunolabeled separately with the following combinations: (1) mouse anti-GFP (1:500; Sigma) and rabbit anti-B-myb (1:200; Santa Cruz Biotechnology), (2) mouse anti-GFP (1:500) and rabbit anti-C-myb (1:200; Santa Cruz Biotechnology), (3) rabbit anti-GFP (1:1000; Clontech, Cambridge, UK) and mouse anti-Tuj1 (1:2000; Covance, Princeton, NJ), (4) rabbit anti-GFP (1:1000) and mouse anti-nestin (1:2000; rat-401 from the Developmental Studies Hybridoma Bank antibody collection, University of Iowa, Iowa City, IA), or (5) rabbit anti-GFP (1:1000) and mouse anti-GFAP (1:500; Chemicon, Temecula, CA) in 10% non-immune goat serum and 0.3% Triton X-100 overnight, followed by secondary labeling with goat FITC-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibodies (Alexa; Molecular Probes, Eugene, OR) at 1:5000 for combinations 1 and 2, and with goat rhodamine-conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies for combinations 3–5. Quantitative assessment of staining was performed in a blinded manner.

**Western blotting.** Western blotting was performed as described previously (Liu and Greene, 2001a).

**Survival assays and counting of apoptotic nuclei.** Survival assays and counting of apoptotic nuclei were as described previously (Liu and Greene, 2001a). Quantitative assessment of survival and death was performed in a blinded manner. All experiments were performed at least in triplicate, and results are reported as means ± SEM. Student’s t test was performed as unpaired, two-tailed sets of arrays and presented as probability (p) values.

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**Figure 1.** siB-myb and antisense B-myb constructs downregulate B-myb expression. A, Coexpression with either siB-myb or antisense B-myb downregulates expression in HEK293 cells of human/rat B-myb. pU6 and pCDNA are control, empty plasmids. B, Expression of either siB-myb or antisense B-myb downregulates endogenous B-myb expression in cycling PC12 cells. siLuc and pCDNA are control plasmids. Transfection efficiency was ~60 – 80%. C, D, Antisense B-myb represses the upregulation of B-myb in cortical neurons treated with camptothecin (campto). Percentage of stained cells in this and subsequent figures pertains to the population of transfected neurons (as indicated by staining for GFP). E, F, siB-myb represses the upregulation of B-myb in cortical neurons treated with camptothecin. Scale bars, 10 µm.
Results

Apoptotic stimuli elevate endogenous B- and C-myb in neurons, and this is blocked by B- and C-myb siRNA and antisense constructs

We reported previously that B- and C-myb genes are repressed in viable neurons and de-repressed in dying neurons (Liu and Greene, 2001a). To investigate whether B-myb plays a required role in neuronal apoptosis, we first prepared and examined the capacities of siB-myb and antisense B-myb constructs to downregulate the expression of rat B-myb. As shown in Figure 1A, coexpression of siB-myb (lane 2) effectively downregulated the expression in HEK293 cells of human B-myb that was mutated so that the siRNA target region matches the rat B-myb sequence (compare lane 2 with control lane 1). The capacity of the antisense B-myb to depress the level of coexpressed human/rat mutant B-myb was similarly confirmed in HEK293 cells (Fig. 1A, compare lane 4 with control lane 3). To examine the abilities of the two constructs to downregulate endogenous B-myb, we cotransfected them with pCMS-EGFP (a control for transfection efficiency) into cycling PC12 cells and analyzed B-myb expression by Western blotting. As a control, empty vector was used in the case of antisense B-myb and a scrambled siRNA (siLuc) in the case of siB-myb. Approximately 60–80% of the cells were transfected under these conditions. As shown in Figure 1B, expression of either siB-myb or antisense B-myb significantly reduced endogenous B-myb expression.

Next, we cotransfected siB-myb, antisense B-myb, or a control vector together with pCMS-EGFP into cultured cortical neurons with or without 12 hr exposure to the proapoptotic DNA-damaging agent camptothecin. The transfected cells were then immunostained to reveal B-myb expression. As illustrated in Figure 1C–F, for control cultures (vector alone or siLuc), little or no staining was detectable without camptothecin exposure, whereas there was a high level of staining in ~75% of the neurons after the treatment. This is consistent with our previous report that B-myb is upregulated in neurons in response to apoptotic stimuli (Liu and Greene, 2001a). We additionally found that antisense B-myb and siB-myb each very significantly suppressed the induction of endogenous B-myb by camptothecin exposure (Fig. 1C–F). Similar observations were made for another apoptotic paradigm, NGF-deprivation in neuronal PC12 cell cultures (data not shown).

We used the same strategies to block induction of C-myb in neurons. As shown in Figure 2, siC-myb and antisense C-myb both suppress expression of cotransfected rat/human mutant C-myb in HEK293 cells (Fig. 2A) and of endogenous C-myb in cycling PC12 cells (Fig. 2B). Consistent with our previous report (Liu and Greene, 2001a), immunostaining revealed a substantial increase in endogenous C-myb expression in cultured cortical neurons after 12 hr exposure to camptothecin (Fig. 2C–F). This increase was substantially suppressed by both antisense C-myb (Fig. 2C,D) and siC-myb (Fig. 2E,F). Here again, there were similar results in NGF-deprived PC12 cell cultures (data not shown).

Downregulation of B-myb and C-myb promotes neuronal survival in multiple apoptotic paradigms

We next examined the effects of B- and C-myb downregulation on neuronal cell survival in a variety of apoptotic paradigms. Figure 3A shows that antisense B-myb and antisense C-myb each provided substantial protection against death evoked by NGF deprivation in PC12 cell cultures. Cotransfection of both antisense constructs increased survival almost to the level observed in the presence of NGF, siB-myb and siC-myb as well as the combination of the two also protected PC12 cells against NGF withdrawal (data not shown) and against camptothecin exposure (Fig. 3B). These constructs additionally suppressed death of cultured cortical neurons induced by camptothecin (Fig. 3C); similar results were obtained with antisense B-myb and antisense
C-myb (data not shown). Downregulation of mybs with siB-myb and siC-myb (Fig. 3D) or antisense B-myb and antisense C-myb (data not shown) greatly enhanced survival of cultured sympathetic neurons for at least 3 d after NGF deprivation. This effect could also be seen by quantifying the numbers of apoptotic nuclei in transfected experimental and control cultures (Fig. 3E and data not shown).

An intriguing aspect of our findings was that similar levels of protection were obtained with constructs designed to interfere with expression of either B-myb or C-myb. Moreover, in most instances, there was only an incremental increase in survival when reagents against both were used at the same time. B- and C-myb bind to similar DNA motifs (Rushton and Ness, 2001), and it has been reported that both B- and C-myb are subject to positive autoregulation (Nicolaides et al., 1991; Sala et al., 1999). Such observations raised the possibility that downregulation of either B- or C-myb might cause cross-downregulation of the other. To test this, we repeated the experiments shown in Figure 1, E and F, in which cortical neurons were transfected with siB-myb or siLuc and treated with or without camptothecin for 12 hr. However, in this case, the cells were stained with antibodies against C-myb. As shown in Figure 3F, camptothecin greatly induced C-myb staining as anticipated. Significantly, siB-myb (but not siLuc) substantially repressed this induction. In contrast, expression of a control antigen (Cdk4) was not affected. Similar results were achieved when we performed the converse experiment of exposing the neurons to siC-myb and staining for B-myb (Fig. 3G). These findings indicate that interfering with expression of either B- or C-myb results in downregulation of the other. Such observations appear to explain why siRNA or antisense constructs against either protein are equally effective.

**Downregulation of B-myb and C-myb mitigates the apoptotic effect caused by E2F de-repression**

We showed previously that E2F1(1–368), a truncation mutant of E2F1 lacking an activation domain, causes depression of myb expression in neurons and efficiently induces their death (Liu and Greene, 2001a). E2F1(1–374) (Phillips et al., 1997) is a similar E2F1 truncation mutant without an activation domain that also promotes de-repression of mybs (data not shown) and that induces death of cortical neurons and neuronal PC12 cells (Fig. 4). As shown in Figure 4A, expression of E2F1(1–374) brought about rapid death of neuronal PC12 cells so that only ~20% survived 2 d after transfection and 3% 3 d after transfection. Cotransfection with siB-myb and siC-myb increased survival to ~60–70% on day 1 and 25% on day 2. This increase in survival was mirrored by a substantial drop in the proportion of apoptotic nuclei present in the cultures (Fig. 4B). To extend this analysis to cortical neurons, E2F1(1–374) was cotransfected with either siLuc, siB-myb, or siC-myb. Again, E2F1(1–374) induced massive death, and this was significantly abrogated by myb downregulation (Fig. 4C). These findings support the idea that B- and C-myb are downstream mediators of death induced by E2F de-repression.

**Discussion**

Past and the present findings demonstrate that apoptotic stimuli such as NGF deprivation and DNA-damaging agents lead to elevated expression of B- and C-myb in both peripheral and CNS neurons (Estus et al., 1994; Liu and Greene, 2001a; Besirli et al., 2003). The major aim of the present study was therefore to assess whether B- and C-myb play required roles in these paradigms of neuron death. To this end, we prepared both antisense and
indicating that both B- and C-myb are required for mediation of apoptosis. We further observed that our constructs provided substantial protection in our apoptotic models, thus proposing as novel targets for cancer therapy; our results suggest that such targeting may also be considered for therapeutic blockade of neuron cell death.

Figure 4.

Downregulation of B-myb and C-myb protects neuronal cells from death caused by E2F de-repression. A, B, Coexpression of siB-myb or siC-myb increases survival (A) and decreases apoptotic nuclei (B) in neuronal PC12 cells expressing E2F1(1–374). A, Significance comparisons with E2F1(1–374) alone on the same day, *p < 0.02; **p < 0.005. B, Significance comparisons with E2F1(1–374) alone, *p < 0.02; **p < 0.005. C, Coexpression of siB-myb or siC-myb promotes survival of cortical neurons expressing E2F1(1–374). Significance comparisons with siLuc coexpressed with E2F1(1–374) as indicated on the same day, *p < 0.01; **p < 0.002.

siRNA constructs targeted to B- and C-myb and demonstrated that each approach effectively blocks induction of these proteins by apoptotic stimuli. We further observed that our constructs provided substantial protection in our apoptotic models, thus indicating that both B- and C-myb are required for mediation of neuron death.

Although overexpression of B- or C-myb may induce death, it has also been reported that dominant-negative (d/n) forms of myb promote apoptosis of non-neuronal cells (Taylor et al., 1996; Lauder et al., 2001; Yi et al., 2002). We also found that d/n myb induces neuron death (Liu and Greene, 2001a). Such findings raise the possibility that neuron survival may require an optimal level of myb expression; on one hand, elevated expression may promote apoptotic death, but on the other hand, a basal level of expression may be required for viability. Consistent with this, we detected both B- and C-myb in viable neurons (Liu and Greene, 2001a), and C-myb is widely and constitutively expressed in brain (Shin et al., 2001). Moreover, we observed neuron death with application of myb antisense and siRNA constructs at concentrations as little as 2.5-fold higher than those that effectively protected neurons from apoptotic stimuli (our unpublished results). In this regard, it may be relevant that Bcl-2, the only protein that has also been reported that dominant-negative (d/n) forms of myb induce death, it...


