Viral Proteins E1B19K and p35 Protect Sympathetic Neurons from Cell Death Induced by NGF Deprivation

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Abstract. To study molecular mechanisms underlying neuronal cell death, we have used sympathetic neurons from superior cervical ganglia which undergo programmed cell death when deprived of nerve growth factor. These neurons have been microinjected with expression vectors containing cDNAs encoding selected proteins to test their regulatory influence over cell death. Using this procedure, we have shown previously that sympathetic neurons can be protected from NGF deprivation by the protooncogene Bcl-2. We now report that the E1B19K protein from adenovirus and the p35 protein from baculovirus also rescue neurons.

Other adenoviral proteins, E1A and E1B55K, have no effect on neuronal survival. E1B55K, known to block apoptosis mediated by p53 in proliferative cells, failed to rescue sympathetic neurons suggesting that p53 is not involved in neuronal death induced by NGF deprivation.

E1B19K and p35 were also coinjected with Bcl-Xs which blocks Bcl-2 function in lymphoid cells. Although Bcl-Xs blocked the ability of Bcl-2 to rescue neurons, it had no effect on survival that was dependent upon expression of E1B19K or p35.

PROGRAMMED cell death plays a key role during development of the nervous system (for review see reference 36), although the molecular mechanisms by which neurons die are unknown. Epigenetic factors, such as neurotrophic factors, seem to promote neuronal survival by blocking an intrinsic cell death program (for review see references 26, 38). Recent identification of proteins that can block apoptosis may be used as tools to unravel pathways of cell death. The Bcl-2 protooncogene (2, 44) is the prototype of these anti-death proteins (1, 13, 16, 24, 31, 40), and a family of proteins homologous to Bcl-2 is now emerging (for review see reference 49). Among these, the Bcl-X gene is the most homologous to Bcl-2 (6) and encodes two splice variants termed Bcl-Xl and Bcl-Xs. Bcl-Xs lacks a 63–amino acid region that is conserved between different Bcl-2 family members. Whereas Bcl-Xl has anti-apoptotic function, Bcl-Xs inhibits the ability of Bcl-2 to enhance the survival of trophic factor–deprived cells (6).

Other anti-apoptotic proteins, with no obvious primary sequence homology with members of the Bcl-2 family, have also been characterized. Among these are the E1B19K and E1B55K proteins from adenovirus and the p35 protein from baculovirus.

The E1B gene encodes two major proteins, the 19-kD and 55-kD proteins which cooperate with E1A proteins to allow transformation (3, 5, 34, 45). Although E1A alone is capable of stimulating cell proliferation, this is accompanied by rapid cell degeneration due to apoptosis. The E1B proteins overcome this effect thereby enhancing cell transformation (47). The 19-kD E1B protein can also block the cytotoxic action of tumor necrosis factor or of anti-FAS antibodies (18, 20, 47), both of which induce apoptosis (25, 29). The induction of apoptosis by E1A is p53 dependent, and both E1B19K and E1B55K inhibit the apoptotic activity of p53 (12, 30, 39). The E1B55K protein interacts with p53 and inhibits its activity as a transcription factor whereas the mechanism of p53 inhibition by E1B19K is unknown.

p35 was first characterized in the *Autographa californica* nuclear polyhedrosis virus. The protein has been shown to be necessary to prevent premature cell death of virus-infected *Spodoptera furgiperda* (SF) insect cells (9). The protein is also expressed and used by other strains of baculoviruses (27). It has been shown that p35 transfected in a mammalian neural cell line can block apoptosis induced by the withdrawal of serum or glucose or by calcium ionophore (37). p35 also prevents apoptosis and rescues a ced-9 mutant in the nematode *C. elegans* (43).

These anti-apoptotic proteins regulate key processes controlling apoptosis and are therefore likely to provide important insights into steps underlying cell death. Here we have tested the effect of E1A, E1B, and p35 on the survival of post-

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mitotic sympathetic neurons cultured in the presence or absence of NGF (14, 32). We show that while E1A or E1B55K fail to affect neuronal survival, E1B19K and p35 block apoptosis induced by NGF withdrawal. The mode of action of these proteins may be different from that of Bcl-2 since coinjected Bcl-Xs cannot block the actions of p35 or of E1B19K. Finally, we show that Bcl-Xs expression does not induce cell death of neurons in the presence of NGF, suggesting that Bcl-2 is not an essential component of the NGF survival pathway.

Materials and Methods

Sympathetic neuron cultures: sympathetic neurons from superior cervical ganglia (SCG)[1] were cultured as previously described (17). Briefly, SCG from newborn rats were dissociated in dispase for 30 min. Neurons were then mechanically dissociated and plated at a low density ($10^4/cm^2$) in 3.5-cm petri dishes coated with collagen. During the first 4 d of culture, neurons were cultured in Leibowitz medium, 5% rat serum, 0.75 μg/ml 7S NGF (Boehringer Mannheim Corp., Indianapolis, IN) and 10^{-5} M arabinosine cytosine C (ARAC). On day 4, after plating culture medium was renewed but ARAC was omitted. Neurons were microinjected between day 5–7 of culture. 3 h after injection, neurons were fed with fresh medium without NGF, 2.5% rat serum and antibodies to NGF (Boehringer Mannheim Corp.).

Microinjections

Sympathetic neurons were microinjected 5–7 d after plating as previously described (17). Before injection, cultures were washed with fresh medium. All neurons within an area that was marked on the bottom of the culture dish were microinjected with a low pressure microinjection system (automatic injector Inject + Matic, Geneva). The DNA constructs were diluted in water, 0.5 mg/ml FITC-dextran, at concentrations of 0.01–0.1 mg/ml. 3 h after injection neurons were counted to determine the initial size of the injected population. Approximately 85% of injected neurons survived the stress caused by injection.

Survival Assay

For determination of cell viability, the dye 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), which is converted to insoluble purple formazan crystals by the mitochondrial dehydrogenases of living cells, was added to the culture medium at 0.5 mg/ml. Cells were incubated at 37°C for 20 min and positive neurons counted under light microscopy (35). HOECHST dye #33342, a DNA minor groove-binding ligand was added to the culture medium simultaneous to MTT. Chromatin staining was viewed under ultraviolet fluorescence.

Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde in PBS, permeabilized for 10 min with 0.2% Triton X-100 in PBS, and incubated for 2 h with monoclonal antibodies to E1A (48), E1B19K, or Bcl-2 (Cambridge Research Laboratories, Wilmington, DE) followed by FITC-conjugated goat anti-mouse antibody.

Plasmid Constructions

Constructions of E1B19K, E1B55K, and E1A expression vectors has been described previously (45–47). Construct for Bcl-2 expression was described in (17).

The baculovirus P35 gene was cloned by PCR using synthetic oligonucleotides based upon its published sequence (16). Tails including ClaI sites were added to each end resulting in 5'-TTAATTAATTAAATCGATT-ATGTGTGTAATTTTTCCGGTAGA for the amino terminus and TACTGA-TATTAAATCGATTTATTTAATTGTGTTTAATATTACATTA for the carboxy terminus. PCR was conducted for 20 cycles using 20 ng of purified wild-type baculovirus DNA as template. The amplified DNA fragment was

digested by ClaI endonuclease and the resulting fragment of 900 bp inserted into the unique ClaI site present downstream of the hCMV promoter of the pEE12 derivative. Clones with the insert in the two orientations were selected. The sequence of the PCR product was identical to the p35 sequence published by Friesen and Miller (1987) (16).

The human Bcl-Xs cDNA was cloned by PCR using RNA from human thymus and synthetic oligonucleotides based upon its published sequence (6). PCR was conducted for 35 cycles using the following oligonucleotides: GAGAATCACTAACCAGAGAC for the amino terminus and AGGTGGTG-TAGGAGGCAGGT for the carboxy terminus. The PCR product was amplified a second time using the following oligonucleotide: TTTGGACAA-TGGACTGGTTGA for the 5' end and CTGGTCTGTGACTGGTAGGT at the 3' end. Two DNA fragments of 760 and 572 bp were amplified and subcloned in the SmaI restriction site of pBluescript (Stratagene, La Jolla, CA) and sequenced. The 760-bp long fragment corresponded to Bcl-Xl and the 572-bp fragment was identical to Bcl-Xs. Bcl-Xs was excised from pBluescript with EcoRI and XbaI and subcloned in pcDNA 1 (Invitrogen, San Diego, CA). All plasmid DNA used for microinjections were purified on a cesium chloride gradient.

Results

SCG Neurons Depend on NGF for Their Survival

Neurons dissociated from superior cervical ganglion of newborn rats were maintained in culture for 5–7 d in the presence of NGF. Under these conditions, they maintained phase bright cell bodies and a dense neurite network (Fig. 1 A). The viability of neurons was demonstrated by a metabolic reduction of MTT (Fig. 1 B), which stains purple in the cytoplasm of cells with functional mitochondria (35) and a nuclear-HOECHST dye staining (Fig. 1 C). 24 h after removal of NGF from the culture medium, the neurons still displayed bright cell bodies but their cytoplasm was slightly reduced in size and appeared granular. In particular, the nucleus and nucleolus were less visible (not shown). After 48 h in the absence of NGF, most neurons were MTT negative and displayed nuclear condensation which is a hallmark of apoptosis (Fig. 1, D, E, and F) (28, 50). Nuclear pyknosis seemed to precede loss of mitochondrial function as a small percentage of neurons that displayed condensed nucleus were still MTT positive (not shown). Atrophy of the cell bodies was followed by neurite disintegration.

Effects of E1B and p35 on the Survival of Sympathetic Neurons

cDNAs encoding E1B19K, E1B55K from adenovirus under control of the CMV promoter (45), and p35 from baculovirus also under control of the CMV promoter, were used to assess the ability of these proteins to regulate apoptosis. The purified DNA was microinjected into the nucleus of 5–7 d-old sympathetic neurons. 3 h after microinjection, NGF was removed and antibodies against NGF added to the culture medium. In all experiments, 100% cell survival referred to the number of living neurons counted 3 h after NGF deprivation. 2 d later, less than 10% of neurons that were not injected or were injected with control DNA survived. We repeatedly observed that neurons that were microinjected with only FITC-dextran in water survived better during the first 24 h after NGF deprivation compared to uninjected neurons. The beneficial effects due to microinjection per se never lasted more than 24 h and by 48 h after injection the survival of microinjected and uninjected neurons was not different (this observation is reported in Fig. 6). At that time, ~10% of neurons injected with a control DNA solution sur-
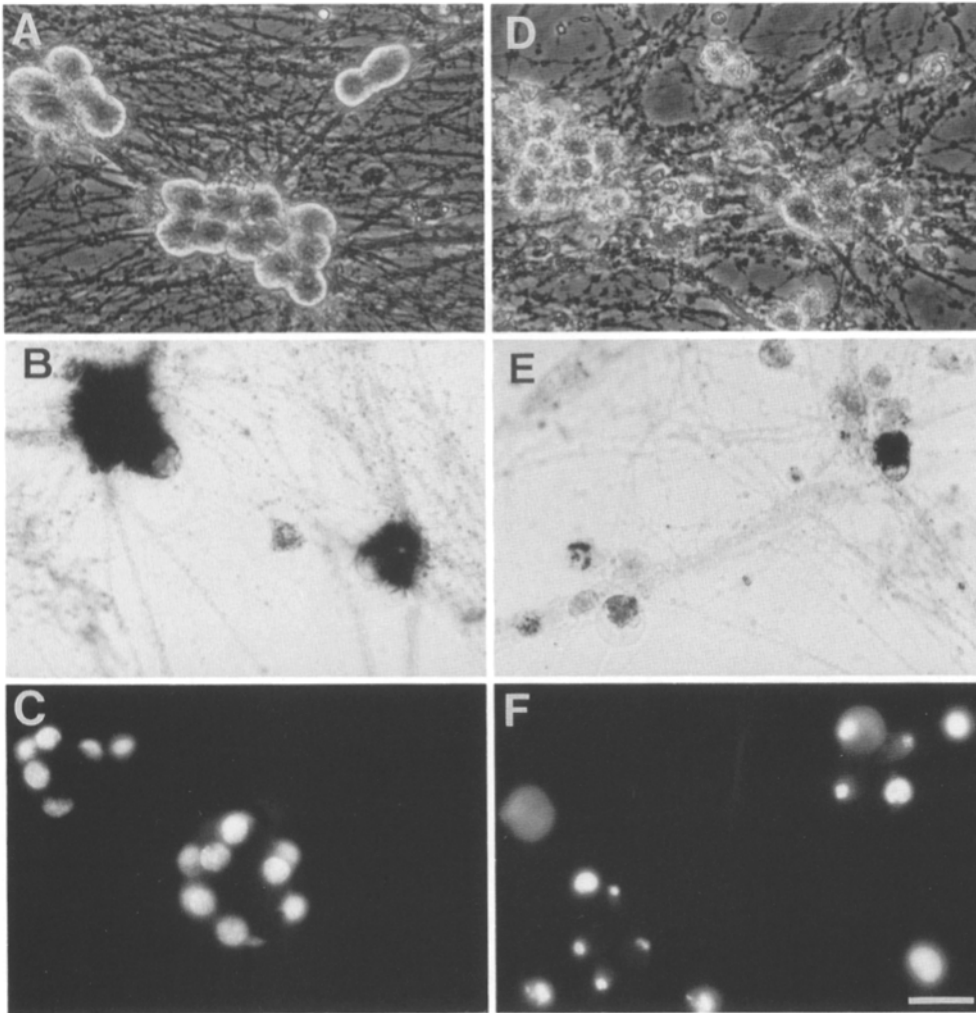
Figure 1. Morphology of sympathetic neurons undergoing apoptosis after NGF deprivation. Neurons from cervical superior ganglia of newborn rats were maintained in culture for 5–7 d in the presence of NGF. Under these conditions, they maintained phase bright cell bodies (*A*). MTT and HOECHST staining confirmed their viability (*B* and *C*). 48-h after NGF deprivation, the majority of neurons were dead. Their cell bodies and neurites were disintegrated (*D*). HOECHST staining revealed nuclear condensation and the MTT test an absence of mitochondrial function (*E* and *F*). Bar, 50 μm.

vived NGF deprivation. In contrast, ~60% of neurons injected with E1B19K (Fig. 2 *A* and Fig. 3) or p35 (Fig. 2 *B*) survived in the absence of NGF. These neurons could be maintained alive for at least 7 d in the absence of NGF. Their cell body and neurites were however atrophied (Fig. 3). These observations are similar to those we previously made for Bcl-2 (17). This suggests that these anti-apoptotic proteins display survival but not trophic effects.

Neither E1B55K, another adenoviral protein, nor pm7fs, a frameshift mutant of E1B19K (47), had beneficial effects on neuronal survival (Fig. 2 *A*).

### Bcl-Xs Can Inhibit the Ability of Bcl-2 but Not of E1B19K or p35 to Prevent Apoptotic Cell Death

Boise et al. (6) have recently reported that coexpression of Bcl-2 with Bcl-Xs, a splice variant of Bcl-X, inhibits Bcl-2 from preventing apoptotic death of FL5.12 cells upon growth factor removal. We have tested whether Bcl-Xs could block the anti-apoptotic function of Bcl-2, E1B19K, and p35 in SCG neurons. Bcl-2, E1B19K, and p35 were injected at a concentration of 0.01 μg/μl with increasing concentrations of Bcl-Xs ranging from 0 to 0.1 μg/μl. β-galactosidase was used as a control for Bcl-Xs. We found that Bcl-Xs inactivated Bcl-2 function in a dose-dependent fashion (Fig. 4 *A*). A
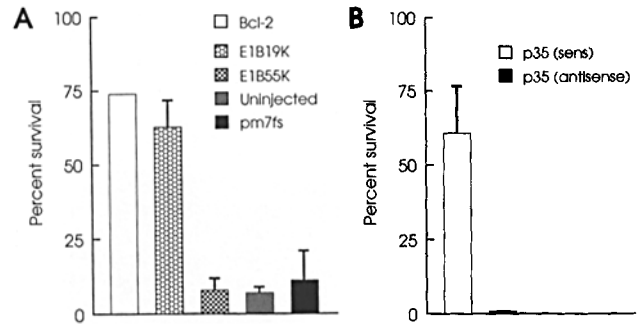
Figure 2. Effects of overexpression of E1B19K, E1B55K, and p35 on neuronal survival. 5–7 d cultured sympathetic neurons were microinjected with different plasmids encoding E1B19K and E1B55K adenoviral proteins and p35 protein from baculovirus. pm7fs, a frame shift mutant of E1B19K and a plasmid containing p35 cDNA in an antisense orientation were used as controls. For each experiment, different pools of neurons (between 100 and 200) contained in rectangles labeled on the bottom of 3.5-cm petri dishes were microinjected with different plasmids. 3 h after microinjection neurons were deprived of NGF. Neuronal survival was assayed 48 h later and is expressed as the percentage of neurons at the time of NGF deprivation. Results are mean ± SE for 6 and 2 experiments in *A* and *B*, respectively. In *A*, we have included the result of a microinjection with Bcl-2 that was performed in one of the six experiments.
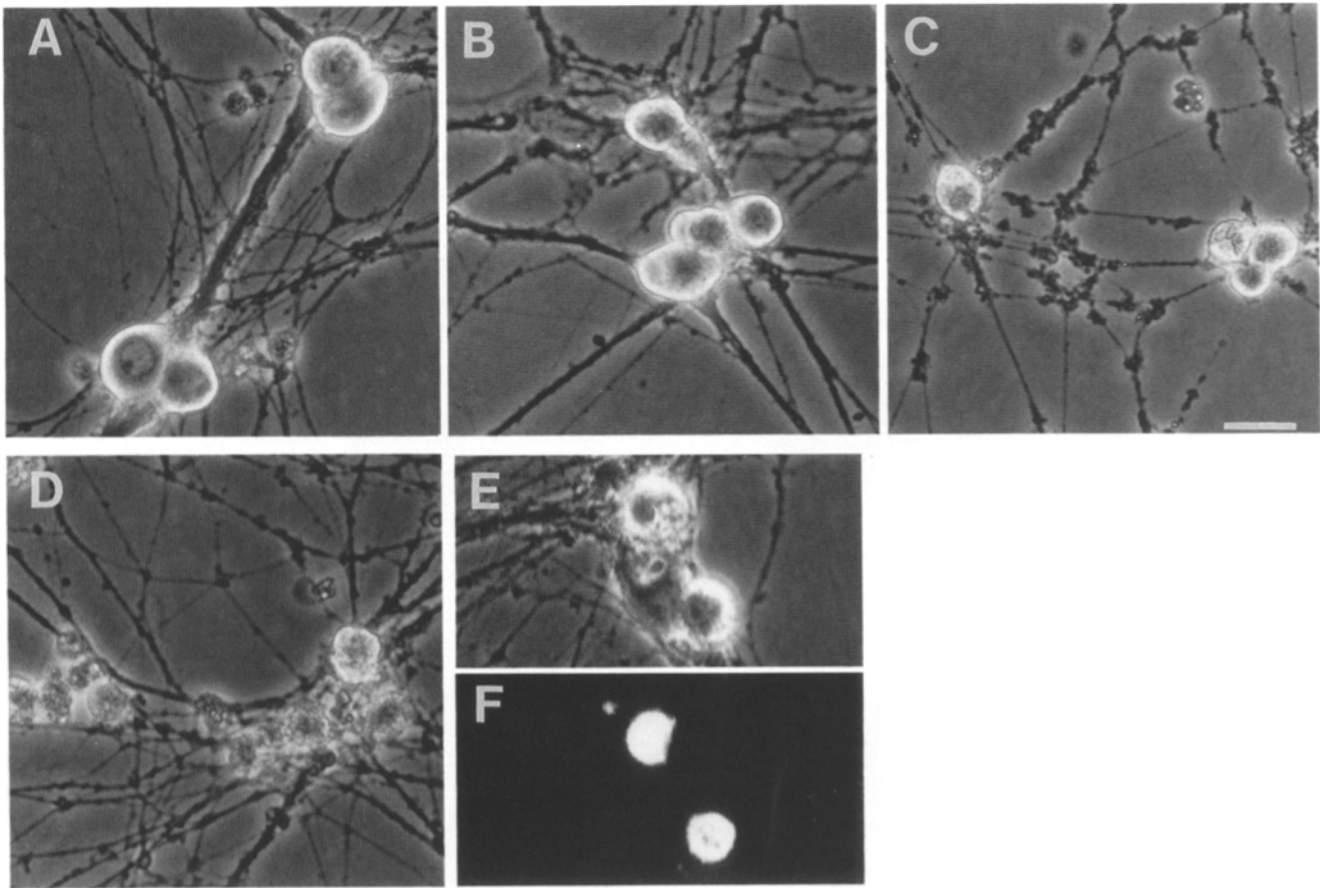
Figure 3. Appearance of E1B19K-microinjected neurons after NGF deprivation. Neurons have been microinjected with E1B19K expression vector and deprived of NGF. Their morphology was analyzed after 2 d (*B*) and 7 d (*C*) and compared to that of neurons cultured in the presence of NGF (*A*) or uninjected neurons deprived of NGF for 2 d (*D*). Expression of E1B19K was assessed by immunostaining (*E*, phase microscopy; *F*, fluorescence microscopy). Bar, 30 μm.
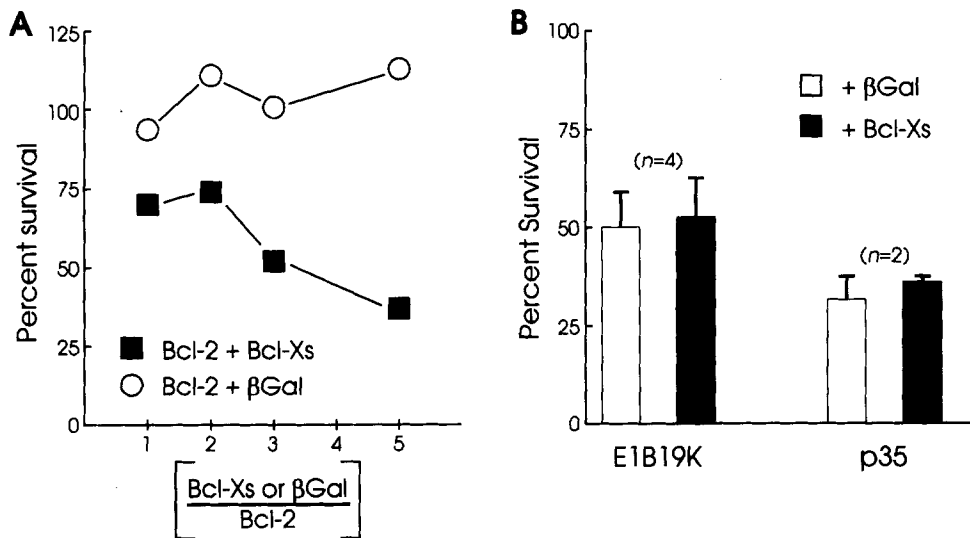
Figure 4. Effects of Bcl-Xs on the rescuing effects of Bcl-2, E1B19K, and p35. (*A*) Sympathetic neurons were microinjected with expression vectors for Bcl-2 and Bcl-Xs. An expression vector for β-galactosidase was used as a control for Bcl-Xs. Bcl-2 alone was also tested in these experiments. The ratio of concentrations of Bcl-Xs or β-galactosidase over Bcl-2 varied from 1 to 5. Neurons were assayed for survival 48 h after NGF deprivation. Results represent the percentage of neuronal survival promoted by Bcl-2 alone considered here as 100% survival. In three independent experiments we found that the number of sur-

viving neurons coinjected with Bcl-2 and Bcl-Xs was reduced by 41% ± 3 compared to the number of neurons injected with Bcl-2 and β-galactosidase. (*B*) The effects of Bcl-Xs were also tested on the survival effects of E1B19K and p35. Neurons were coinjected with expression vectors for E1B19K or p35 and Bcl-Xs. The DNA concentration of Bcl-Xs expression vector was in a 10-fold excess over that of E1B19K or p35 expression vector. The results are the mean (*bar height*) and standard error (*error bar*) of *n* experiments. Between 100 and 200 neurons were injected in each experiment.
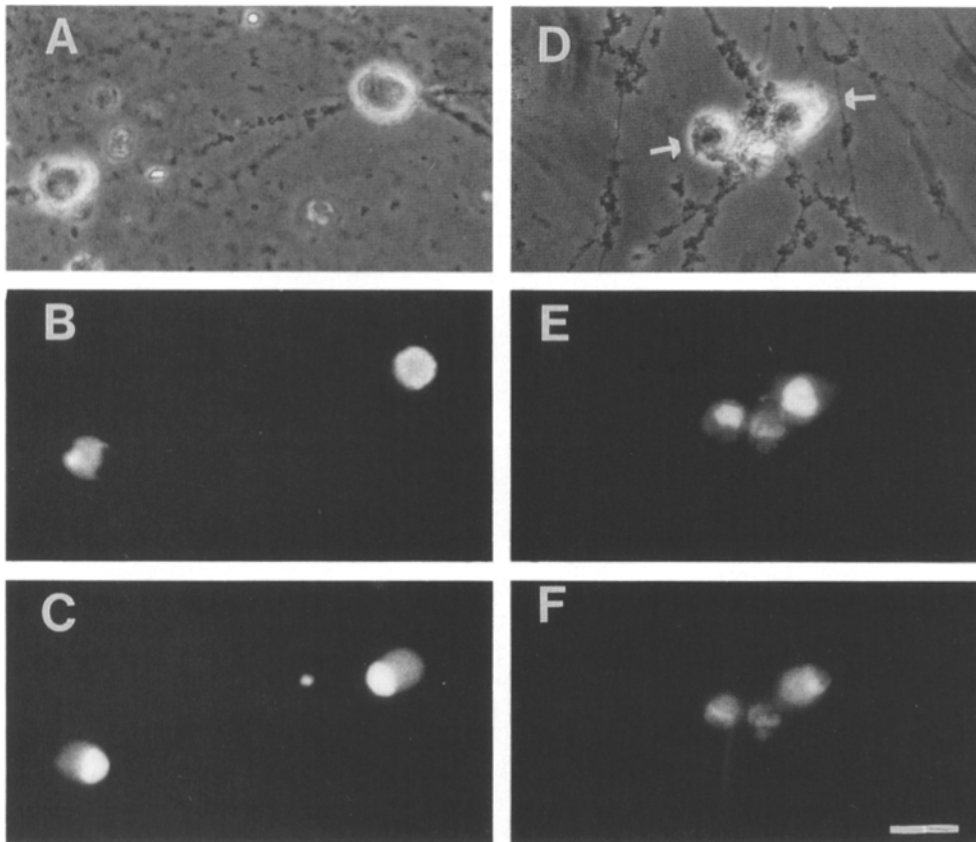
Figure 5. Neurons coexpressing Bcl-2 and Bcl-Xs undergo programmed cell death after NGF deprivation. Neurons have been coinjected with expression vectors for Bcl-2 and β-galactosidase (*A*, *B*, and *C*) or Bcl-2 and Bcl-Xs (*D*, *E*, and *F*) and deprived of NGF. Three days later, they were immunostained for Bcl-2 (*B* and *E*) and their nucleus visualized by HOECHST staining (*C* and *F*). In *D*, three neurons undergo programmed cell death; two of them indicated by arrows display nuclear condensation despite Bcl-2 overexpression. Bar, 20 μm.

large proportion of neurons overexpressing Bcl-2 and Bcl-Xs displayed nuclear condensation visualized by HOECHST staining. A picture of such a neuron is shown in Fig. 5. In contrast, Bcl-Xs had no effect on the function of E1B19K and p35; even when the concentration of the vector for Bcl-Xs expression was in a 10-fold excess over that containing p35 or E1B19K, no adverse effect was observed on the protective function of both proteins (Fig. 4 *B*). To rule out the possibility that Bcl-Xs expression may have altered Bcl-2 expression, we measured the level Bcl-2 in the presence or absence of coinjected Bcl-Xs, by immunostaining using a confocal microscope. Immunofluorescence intensity of neurons injected with expression vectors for Bcl-2 alone was not significantly different from that of neurons injected with Bcl-2 together with Bcl-Xs or β-galactosidase. The fluorescence levels detected and expressed in arbitrary units were: Bcl-2: 208 ± 43; Bcl-2 and β-galactosidase: 193 ± 55; Bcl-2 and Bcl-Xs: 188 ± 59, mean ± SD for 20 neurons analyzed. Therefore the inhibition of Bcl-2 activity observed upon coinjection with Bcl-Xs cannot be accounted for by a drop in Bcl-2 expression.

Bcl-Xs Does Not Interfere with Survival Effects of NGF

Neurons injected with Bcl-Xs alone were left in the presence of NGF for up to a week. Under these conditions, Bcl-Xs had no deleterious effect on neuronal survival (not shown). Also the kinetic of cell death after NGF withdrawal was not detectably affected (Fig. 6).

E1A Does Not Affect Neuronal Survival

Previous studies have shown that E1A expression is

detrimental to cell viability. We have tested whether this protein may have a similar effect on postmitotic neurons. E1A expression was detected after DNA injection using a monoclonal antibody to the protein. The E1A protein was only localized in the nucleus but not within the nucleolus (Fig. 7 *A*). Neurons overexpressing the E1A protein did not show any obvious morphological signs of mitosis when cultured
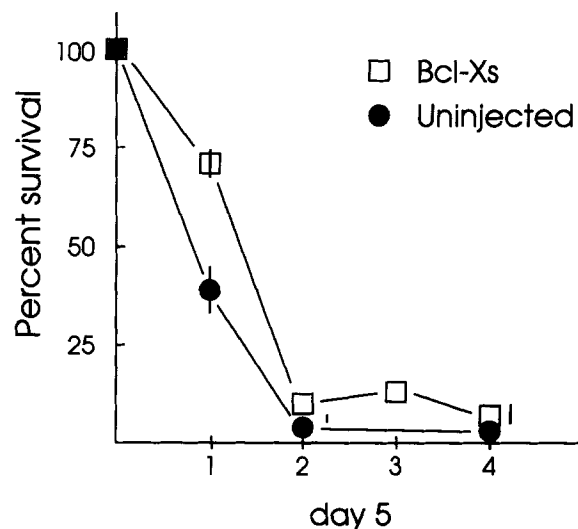
Figure 6. Effects of Bcl-Xs on neuronal survival. Sympathetic neurons were microinjected with expression vectors containing Bcl-Xs and their survival compared to that of uninjected neurons for 4 d after NGF deprivation. Results are mean ± SE for two experiments.

A

B
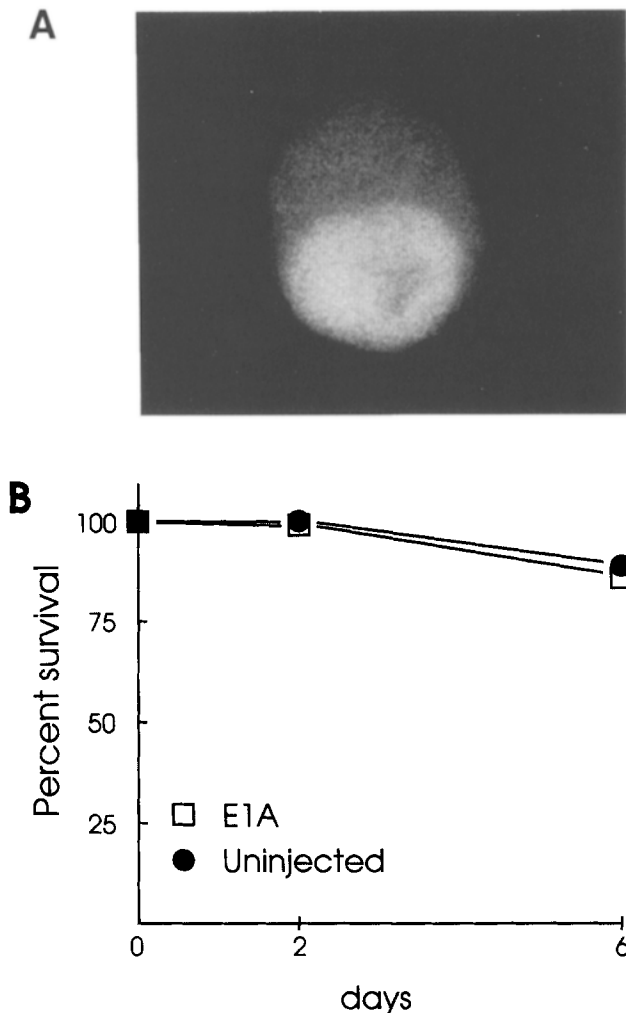
Figure 7. Effects of E1A overexpression on the survival of sympathetic neurons. Sympathetic neurons were microinjected with an expression vector for E1A. (*A*) 24 h later the neurons were immunostained for E1A and analyzed with a confocal microscope. (*B*) Kinetic of survival of neurons overexpressing E1A in the presence of NGF.

in the presence or 24 h after deprivation of NGF. In particular no bromodeoxyuridine incorporation could be detected indicating the absence of DNA synthesis (not shown). Furthermore, cell death in the presence of NGF was not affected by overexpression of E1A (Fig. 7 *B*).

Discussion

Several viral genes such as the baculovirus p35 gene (9, 11, 23), the Epstein-Barr virus latent genes (19, 21, 22), adenovirus E1B (39), and the herpes simplex virus neurovirulence gene (8) encode proteins that can protect cells from apoptosis induced upon viral infection. These genes promote viral intracellular persistence and allow viral replication within the infected cell. In the case of the adenovirus, E1B genes are necessary for transformation since they block apoptosis induced by the oncogenic activity of E1A (47). Here we have used two viral proteins, E1B19K from adenovi-

rus and p35 from baculovirus, as tools to get insights into the mechanisms by which postmitotic neurons die after neurotrophic factor deprivation. We report that independently of viral infection, the two viral proteins can block apoptosis induced by neurotrophic factor deprivation in neurons. These proteins are as efficient as the Bcl-2 protooncogene which protects neurons from apoptosis induced in similar conditions (1, 4, 17, 31).

Bcl-2 can protect a vast array of vertebrate and invertebrate cells against cell death (for review see reference 40). This suggests that the basic mechanisms driving cell death are common to most cells and highly conserved throughout evolution. However, it is noteworthy that Bcl-2 does not protect in every case of apoptosis: it does not rescue target cells from cytotoxic T cell killing, neither does it rescue all cell lines from cytokine deprivation, T cells from Thy-1 antibody induced apoptosis (for review see reference 40), nor chicken ciliary neurons from ciliary neurotrophic factor deprivation (1). Finally, we have evidence that in transgenic mice, overexpression of Bcl-2 does not protect all neurons from naturally occurring cell death (33). Therefore, upon particular stimuli, cells may use pathways that escape Bcl-2 protection. In an effort to sort out cell death pathways it may be very relevant to test whether E1B and p35 are able to block apoptosis in situations where Bcl-2 has failed to inhibit this phenomenon.

Recently, Boise et al. (6) reported the cloning of Bcl-X, a gene highly homologous to Bcl-2. Two splice variants encoding a long and a short form of Bcl-X were described. A long form, Bcl-Xl seems to function interchangeably with Bcl-2. Bcl-Xl mRNA is strongly expressed in tissues containing long-lived cells such as adult brain. The short spliced product, Bcl-Xs, lacks a 63-amino acid region that is highly conserved between different members of the Bcl-2 family. Bcl-Xs has no anti-apoptotic activity but instead inhibits Bcl-2 function (6). Since Bcl-Xs does not interact with Bcl-2, the current hypothesis is that it acts as a dominant negative mutant of Bcl-2 by competing for and blocking effector proteins. Although p35 does not display obvious amino acid sequence homology with Bcl-2 and although the homology between Bcl-2 and E1B19K is limited (7), they could however interact with common effectors. We have used Bcl-Xs to test this hypothesis. Similar to the findings described by Boise et al. (6) using an IL-3–dependent cell line, we found that Bcl-Xs reduced the rescuing effect of Bcl-2 on neurons deprived of NGF. However Bcl-Xs had no influence on the anti-apoptotic activity of p35 and of E1B19K. Although we cannot exclude that the failure of Bcl-Xs to block E1B19K and p35 simply reflects a higher activity of these two proteins over that of Bcl-2, these findings suggest that E1B19K, p35, and Bcl-2 display different mechanisms of action. In favor of this hypothesis, it was shown that actinomycin D-induced apoptosis in SF-21 cells can be blocked by p35 but not by Bcl-2 nor by E1B19K (10).

Very little is known about the molecules which mediate the surviving activity of NGF. Recently Bcl-2 and Bcl-Xl have been shown to be expressed in neurons during development and in adulthood (6). The ability of these proteins to rescue neurons from neurotrophin deprivation, suggested that they may represent downstream effectors of neutrophins. However Bcl-Xs, which blocks Bcl-2 function, had no deleterious effect on SCG neurons. This suggests that neither Bcl-2

nor Bcl-X are essential components of the NGF survival pathway.

Apoptosis has recently been hypothesized to be the result of aberrant cell-cycle control. In favor of this, Shi et al. (42) have shown that activation of p34cdc2 is required for apoptosis induced by a cytotoxic granule protease. Another common feature between apoptosis and mitosis has been described by Freeman et al. (15): Cyclin D1, a protein previously shown to be essential for progression through the G1 phase of the cell cycle, is selectively induced in SCG neurons undergoing cell death after NGF deprivation. In relation to this, we have examined the effect of E1A overexpression in postmitotic neurons. E1A expression is sufficient to initiate proliferation of primary baby rat kidney cells and focus formation. The ability of E1A to stimulate cellular DNA synthesis accompanies the induction of apoptosis (46). After a discrete number of divisions, these cells will undergo apoptosis mediated by p53 (12, 47). In neurons, expression of E1A for several days had no deleterious effect on survival. The lack of E1A toxicity in neurons could be due to the incapacity of neurons to replicate DNA. Our results further support the idea that cell death induced by E1A represents only an undesirable side effect of the proliferation induced by the protein and not a toxic effect of the protein per se. The lack of protective effect of E1B55K on neurons deprived of NGF underlines the molecular differences between mechanisms of cell-death in proliferating vs non-proliferating cells. Indeed E1B55K blocks apoptosis of proliferating cells by binding to p53 and directly blocking its activity (41, 51). Sympathetic neurons express p53 (15). The lack of rescuing activity of E1B55K strongly suggests that p53 is unnecessary for neuronal death induced by NGF deprivation.

In summary, we have shown that two viral proteins E1B19K and p35 are capable of rescuing postmitotic neurons from cell death induced upon NGF withdrawal. We also demonstrated that Bcl-Xs antagonizes the rescuing activity of Bcl-2 but not of p35 or E1B19K. Understanding the function of these proteins should allow the identification of key components of neuronal cell death pathways.

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