Development 122, 695-701 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV1047

Bax promotes neuronal survival and antagonises the survival effects of neurotrophic factors

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SUMMARY

Bcl-2, Bcl-x and Bax are members of a family of cytoplasmic proteins that influence cell survival. Whereas increased expression of Bcl-2 or Bcl-x promotes cell survival following withdrawal of survival factors, increased expression of Bax is thought to suppress survival. To investigate the potential roles of these proteins in regulating the survival of developing neurons, we compared the effects of overexpressing these proteins in embryonic neurons deprived of different neurotrophic factors in vitro. Surprisingly, overexpression of Bax rescued populations of sensory neurons deprived of nerve growth factor, as did overexpression of Bcl-2 and two Bcl-x variants, Bcl-x_L and Bcl-x_β. Bax also enhanced the survival of ciliary neurons deprived of ciliary neurotrophic factor, although this effect was short-lived. Whereas Bcl-2 overexpression did not affect the survival response of neurons to neurotrophic factors, Bax overexpression partially inhibited the action of neurotrophic factors. Co-injection of Bcl-2 and Bax expression vectors promoted the survival of neurotrophic factor-deprived neurons if either was in excess, but failed to rescue neurons if they were injected at a 1:1 ratio. Our findings demonstrate that Bax can promote the survival of neurotrophic factor-deprived neurons and that its effect on survival is dominant to that of neurotrophic factors. Our results also argue that the relative amounts of Bcl-2 and Bax are critical in regulating neuronal survival.

Key words: apoptosis, neurotrophic factor, Bcl-2, Bax, chick

INTRODUCTION

Bcl-2 is a 26×10^3 M_r intracellular, membrane-associated protein of vertebrates (Nunez and Clarke, 1994; Davies, 1995) that is homologous to the nematode ced-9 protein which functions as a negative regulator of cell death (Hengartner & Horvitz, 1994). Experimental overexpression of Bcl-2 prevents the death of several cytokine-deprived haematopoietic cell lines following cytokine withdrawal (Vaux et al., 1988; Nunez et al., 1990) and rescues embryonic neurons deprived of members of the neurotrophin family of survival factors: nerve growth factor (NGF; Garcia et al., 1992), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3; Allsopp et al., 1993). Examination of transgenic mice carrying a *bcl-2* gene that is over-expressed in the immune system (McDonnell et al., 1989) and mice with *bcl-2* null mutations (Nakayama et al., 1993; Veis et al., 1993; Nakayama et al., 1994) have shown that Bcl-2 plays an important role in promoting and regulating the survival of B and T lymphocytes (Linette and Korsmeyer, 1994). Bcl-2 is not, however, universally effective in counteracting signals that induce apoptosis. For example, Bcl-2 overexpression does not protect all cytokine-dependent haematopoietic cell lines following cytokine withdrawal (Nunez et al., 1990) and does not protect neurons following withdrawal of ciliary neurotrophic factor (CNTF; Allsopp et al., 1993).

Several genes have recently been identified that are homologous with *bcl-2*, including *bcl-x* (Boise et al., 1993), *bax* (Oltvai et al., 1993), *bad* (Yang et al., 1995) and *bak* (Chittenden et al., 1995; Farrow et al., 1995; Klefer et al., 1995). The Bcl-x variant, Bcl-x_L has the highest homology with Bcl-2, and like Bcl-2 is able to prevent apoptosis of IL3-dependent cells following IL3 withdrawal (Boise et al., 1993). Another Bcl-x variant, Bcl-x_β (Gonzalez-Garcia et al., 1994) lacks a C-terminal hydrophobic domain that is necessary for membrane attachment. Mice with a null mutation in the *bcl-x* gene die in utero with extensive apoptosis in the immune and nervous systems (Motoyama et al., 1995). Bax was identified as a Bcl-2-associated protein that has 21% amino acid sequence homology with Bcl-2 (Oltvai et al., 1993). Bax heterodimerises with Bcl-2 and also forms Bax homodimers. Bax overexpression in IL3-dependent cells accelerates apoptosis following IL3 withdrawal and inhibits the death repressor action of Bcl-2 in these cells (Oltvai et al., 1993). Consequently, it is thought that the ratio of Bcl-2 to Bax determines survival or death following an apoptotic signal; excess Bcl-2 leading to survival, excess Bax causing death (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994; Korsmeyer, 1995).

To test the generality of the Bcl-2/Bax rheostat model (Korsmeyer, 1995) and to clarify the role of Bcl-2-related proteins in regulating neuronal survival, we used microinjec-

tion to introduce expression vectors into cultures of embryonic chicken neurons that were deprived of different neurotrophic factors. Four populations of neurons were studied: the proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) which are supported by BDNF (Davies et al., 1986), the cutaneous sensory neurons of the dorsomedial part of the trigeminal ganglion (DMTG) which are supported by NGF (Davies and Lindsay, 1985), the mixed sensory neurons of dorsal root ganglia (DRG) which are supported by NGF or BDNF (Lindsay et al., 1985) and the parasympathetic neurons of the ciliary ganglion which are supported by CNTF, growth promoting activity (GPA) and basic fibroblast growth factor (bFGF) (Barbin et al., 1984; Unsicker et al., 1992; Allsopp et al., 1995). Our findings show that Bax overexpression is capable of promoting survival of neurotrophic factor-deprived neurons and that its action is dominant to that of neurotrophic factors. Our results also suggest that the relative amounts of Bcl-2 and Bax are critical in regulating neuronal survival.

MATERIALS AND METHODS

Neuron culture

Fertile white Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator. After 10 or 12 days incubation (E10 or E12), the median part of the TMN, the dorsomedial part of the trigeminal ganglion (DMTG), lumbar DRG, and ciliary ganglia were dissected from the embryos using electrolytically sharpened tungsten needles (Davies, 1988). After incubation with 0.1% trypsin in calcium and magnesium-free Hank's balanced salt solution (HBBS) for 10 minutes at 37°C, the dissected tissue was washed twice in Ham's F12 medium containing 10% heat-inactivated horse serum (HIHS) and was dissociated by gentle trituration using a siliconised, fire-polished Pasteur pipette. Non-neuronal cells were removed by differential sedimentation through a pre-cooled column of Ham's F14 medium containing 10% HIHS (Davies, 1986). The column fractions containing the neurons were centrifuged at 2,000 ***g*** for 5 minutes and the neurons were plated in 60 mm diameter tissue culture dishes that had been coated with polyornithine (0.5 mg/ml in 0.15 M borate buffer, pH8.7, overnight) and laminin (20 μg/ml in F14 medium, 4 hours). The cells were cultured in 5 ml of Ham's F14 medium supplemented with 10% HIHS, penicillin (60 mg/l), streptomycin (100 mg/l) and 24 mM $NaHCO_3$, with or without neurotrophic factors, at 37ºC in a humidified 4% CO_2 incubator.

Cell microinjection

Neurons were washed (3× 10 ml washes) with warm F12 medium containing 10% HIHS before injection with cell injection pipettes (GD-1, Narishige) held by a Narishige micromanipulator attached to the stage of a Nikon Diaphot inverted microscope. All neurons within an area that was marked on the inside of the culture dish were pressure-injected with constructs at a concentration of 100 μg/ml in phosphate-buffered saline. Each neuron was injected until slight cell swelling was observed which usually took place within a few seconds. Intra-nuclear injection resulted in greater than 95% viability assessed 1 hour post-injection. After injection, the neurons were washed twice with warm F12 medium plus 10% HIHS and twice with warm F14 culture medium plus 10% HIHS and 5% heat-inactivated fetal calf serum (HIFCS) and the number of injected neurons was counted 1 hour after injection (because any neurons that were terminally damaged by the injection procedure would have degenerated by this time). The number of neurons surviving in F14 plus 10% HIHS and 5% HIFCS after 24, 48 and 72 hours after injection was counted and is expressed as a percentage of the post-injection number. To examine if overexpression of Bcl-2, Bcl-x_L, Bcl-xβ and Bax proteins are able to prevent the death of neurotrophic factor-deprived neurons, pSFFV constructs containing mouse *bcl-2* (Nunez et al., 1990), *bcl-x*$_L$ (Gonzalez-Garcia et al., 1994), *bcl-xβ* (Gonzalez-Garcia et al., 1994) or *bax* (Oltvai et al., 1993) cDNAs were injected in parallel cultures of neurotrophic factor-deprived neurons. To control for non-specific effects of the injection procedure, the pSFFV vector without an inserted gene was injected into neurons.

RESULTS

Overexpression of Bcl-2, Bcl-x_L, Bcl-xβ and Bax rescues neurotrophin-deprived sensory neurons

Previous in vitro studies have shown that overexpression of Bcl-2 is able to rescue newborn rat sympathetic neurons deprived of NGF (Garcia et al., 1992) and rescue embryonic chicken sensory neurons deprived of NGF, BDNF or NT3 (Allsopp et al., 1993). To determine if overexpression of Bcl-x_L, Bcl-xβ or Bax is able to rescue neurotrophin-deprived neurons and to compare the effects of overexpressing these proteins with Bcl-2 overexpression, NGF-deprived and BDNF-deprived sensory neurons were injected with expression vectors containing *bcl-2*, *bcl-x*$_L$, *bcl-xβ* and *bax* cDNAs. E12 DRG, E12 DMTG and E10 TMN neurons were purified free of non-neuronal cells and were grown with either NGF (DRG and DMTG neurons) or BDNF (TMN neurons) for 12 hours. They were then deprived of these factors by extensive washing and were microinjected with expression vectors for Bcl-2, Bcl-x_L, Bcl-xβ or Bax, and their survival was compared at intervals with the survival of neurons injected with the pSFFV expression vector without inserted cDNA.

Confirming previous findings (Allsopp et al., 1993), Bcl-2 overexpression prevented the death of many NGF-deprived DRG and DMTG neurons and BDNF-deprived TMN neurons (Fig. 1A-C). Overexpression of Bcl-x_L and Bcl-xβ also rescued neurotrophin-deprived neurons as effectively as Bcl-2 overexpression (Fig. 1A-C). Surprisingly, Bax overexpression also prevented the death of neurotrophin-deprived neurons (Fig. 1A-C). Although Bax overexpression did not rescue as many neurons as Bcl-2, Bcl-x_L or Bcl-xβ overexpression, there were over 3-fold more Bax-injected neurons surviving compared with vector-injected neurons, 24 hours post-injection in cultures of DRG and DMTG neurons deprived of NGF, and the difference between Bax-injected and control-injected neurons increased to between 4 and 6 fold after 72 hours (Fig. 1A and B). Although Bax overexpression did not rescue BDNF-deprived TMN neurons as effectively as NGF-deprived DRG and DMTG neurons (compare Fig. 1C with Fig. 1A,B), there were significantly more Bax-injected TMN neurons surviving 24 hours post-injection compared with control injected neurons (P<0.001, t-test). In addition to the studies carried out on E12 DRG and DMTG neurons shown in Fig. 1A,B, studies using E10 DRG and DMTG neurons gave very similar results (data not shown).

Overexpression of Bax but not Bcl-2, Bcl-x_L, Bcl-xβ transiently rescues a subset of CNTF-deprived parasympathetic neurons

We have previously shown that overexpression of Bcl-2 is ineffective in rescuing embryonic chicken ciliary ganglion

neurons deprived of CNTF (Allsopp et al., 1993). To determine if overexpression of Bcl-x_L, Bcl-xβ or Bax is able to rescue CNTF-deprived ciliary neurons, E12 ciliary neurons were grown with CNTF for 12 hours before washing and injection with expression vectors containing *bcl-2*, *bcl-x$_L$*, *bcl-xβ* and *bax* cDNAs. Ciliary neurons injected with Bcl-2, Bcl-x_L or Bcl-xβ expression vectors died rapidly and there was no significant difference between the number of neurons injected with these constructs or vector alone (Fig. 2) or uninjected CNTF-deprived neurons (not shown). These results suggest that Bcl-2, Bcl-x_L and Bcl-xβ have similar specificity in rescuing neurons deprived of different neurotrophic factors. In contrast, Bax overexpression rescued a small number of ciliary neurons following CNTF withdrawal. Although this effect of Bax was smaller and more short-lived than its effect on NGF-deprived neurons, this effect was consistently observed and was statistically significant at 12 and 24 hours postinjection ($P<0.001$, *t*-test). These findings clearly demonstrate that Bax overexpression does not invariably cause apoptosis, but can rescue neurons following neurotrophic factor deprivation. Moreover, Bax seems to have a broader spectrum of activity among neurons in this respect than Bcl-2, Bcl-x_L and Bcl-xβ.

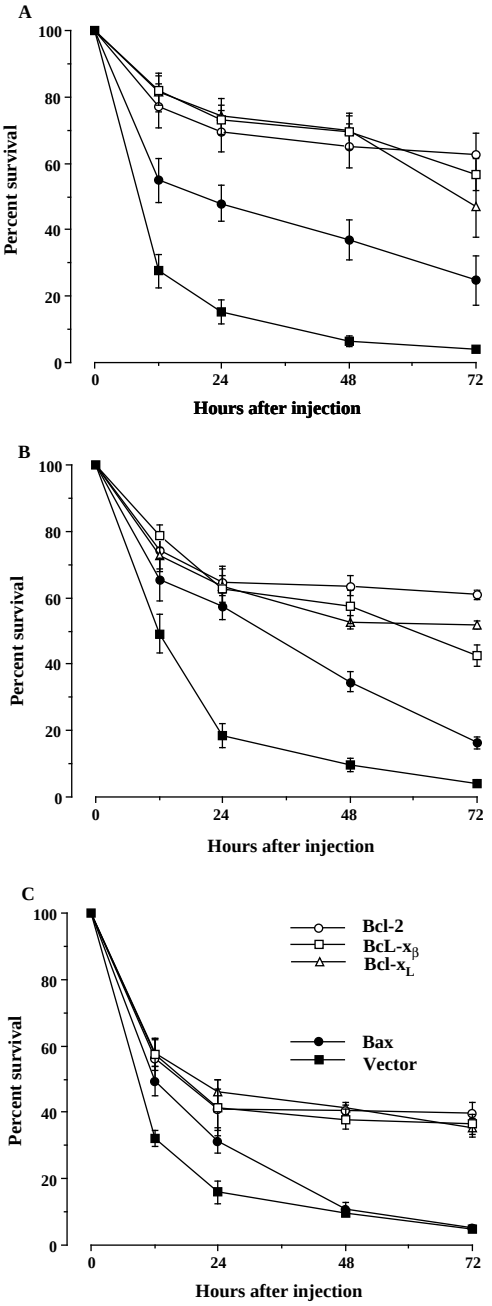

Fig. 1. The effects of injecting NGF-deprived E12 DRG neurons (A), NGF-deprived E12 DMTG neurons (B) and BDNF-deprived E10 TMN neurons (C) with Bcl-2, Bcl-x_L, Bcl-xβ or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml NGF or 5 mg/ml BDNF, the neurons were washed extensively to remove these factors and were injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. Each graph shows the results of three separate experiments. In each experiment, three Petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

Bax overexpression partially inhibits the survival response of neurons to neurotrophic factors

To investigate whether overexpression of Bax modulates the survival response of neurons to NGF and BDNF, we compared the survival of neurons injected with the Bax expression vector grown with and without these neurotrophins. For comparison, neurons were also injected with the Bcl-2 expression vector to determine if this protein affects the neurotrophin survival response differently. DRG, DMTG and TMN neurons were initially grown for 12 hours with either NGF (DRG and DMTG neurons) or BDNF (TMN neurons), then deprived of these neurotrophins and injected with expression vectors for Bcl-2 or Bax, and were then grown with or without the same neu-

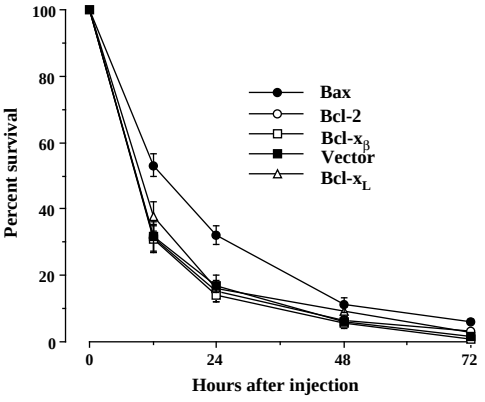

Fig. 2. The effects of injecting CNTF-deprived E12 ciliary neurons with Bcl-2, Bcl-x_L, Bcl-xβ or Bax expression vectors or the pSFFV vector without an insert (control injections). After 12 hours incubation with 5 ng/ml CNTF, the neurons were washed extensively to remove this factor and were injected with expression vectors. The number of neurons surviving at intervals after injection is expressed as the percentage of the initial number of neurons injected. The graph shows the results of three separate experiments. In each experiment, three Petri dishes were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

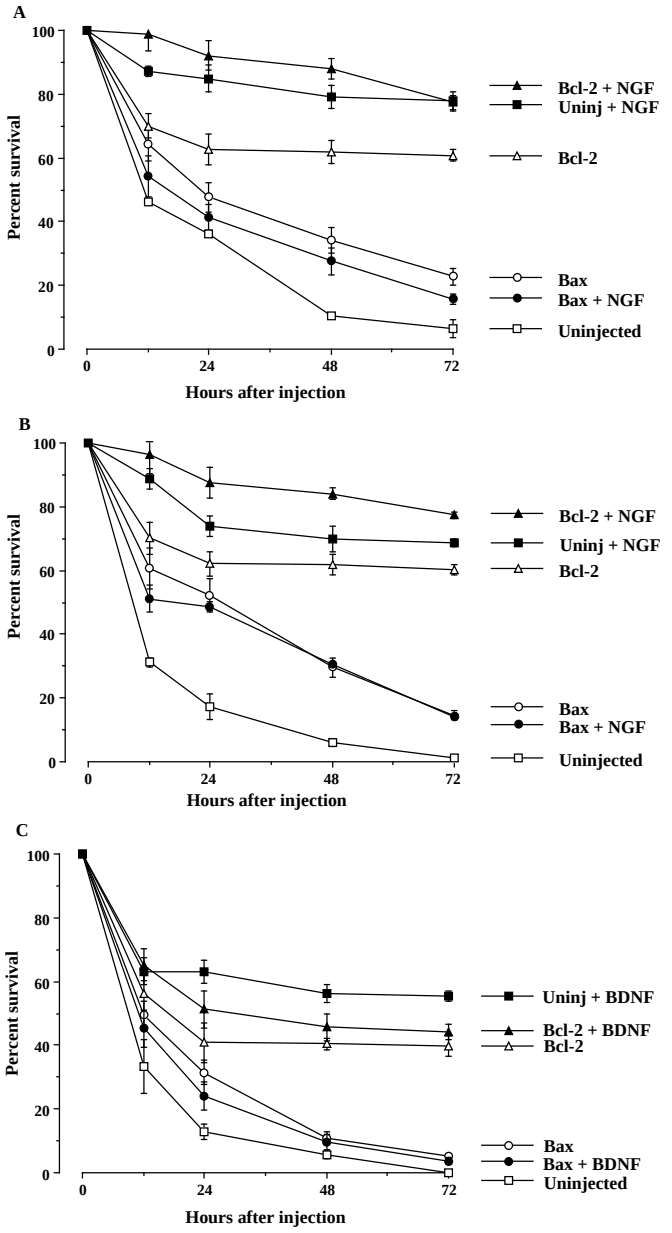

Fig. 3. The effects of overexpressing Bcl-2 and Bax on the survival responses of E12 DRG neurons to NGF (A), E12 DMTG neurons to NGF (B) and E10 TMN neurons to BDNF (C). After 12 hours incubation with 5 ng/ml NGF or 5 mg/ml BDNF, the neurons were washed extensively to remove these factors and were injected with Bcl-2 or Bax expression vectors and were then either resupplemented with the same neurotrophin or grown without neurotrophins for a further 3 days. Parallel cultures of uninjected neurons were also washed and either resupplemented with or without the corresponding neurotrophin. The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three Petri dishesof cells were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

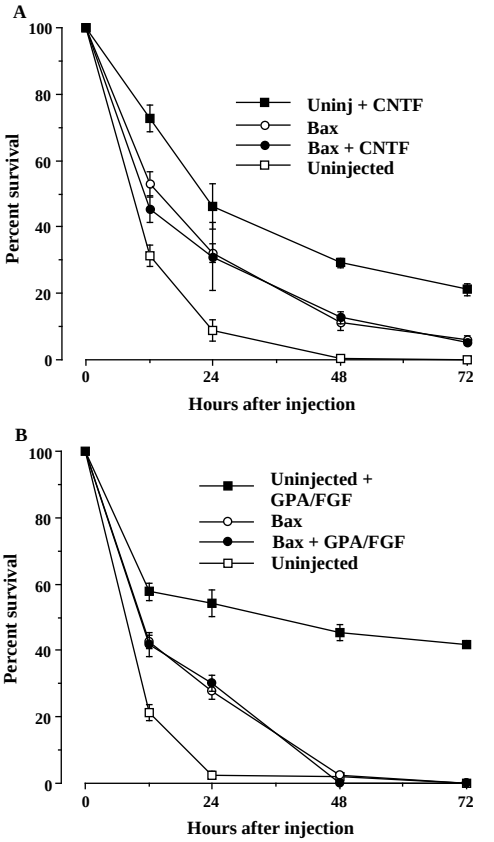

Fig. 4. The effects of overexpressing Bax on the survival response of E12 ciliary neurons to CNTF (A) or GPA plus bFGF (B). After 12 hours incubation with 5 ng/ml of these factors, the neurons were washed and were injected with the Bax expression vector and were then either resupplemented with the same factor(s) or grown in neurotrophic factor-free medium for a further 3 days. Parallel cultures of uninjected neurons were also washed and either resupplemented with or without the same factor(s). The number of neurons surviving at intervals after washing is expressed as the percentage of the initial number of neurons at the time of washing. Each graph shows the results of three separate experiments. In each experiment, three Petri dishes of cells were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

rotrophin for a further 3 days. Fig. 3A-C shows that Bcl-2 overexpression does not interfere with the neurotrophin survival responses of these neurons. Neurons that were injected with the Bcl-2 expression vector and grown with NGF or BDNF survived slightly better than uninjected neurons grown with these neurotrophins. In marked contrast, injection of the Bax expression vector reduced the number of neurons surviving in neurotrophin-supplemented medium to virtually the same number that is sustained by Bax overexpression in the absence of neurotrophins.

Similar studies were carried out to see if Bax also antagonises the survival response of ciliary neurons to neurotrophic factors. As with sensory neurons, Bax overexpression reduced the number of neurons surviving in medium containing CNTF to the same number that are supported by Bax overexpression in medium that does not contain CNTF (Fig. 4A). Because GPA plus bFGF promote better long-term survival of ciliary neurons than CNTF (Allsopp et al., 1995), additional experiments were carried out using these

factors. Here again, Bax overexpression reduced the number of neurons surviving with GPA plus bFGF to the same number that are supported by Bax overexpression in medium without neurotrophic factors (Fig. 4B). The inhibitory effect of Bax overexpression on the neurotrophic factor survival response is particularly evident in these experiments when after 48 hours 50% of the uninjected neurons are still surviving with GPA plus bFGF whereas almost all of the neurons injected with the Bax expression vector are dead in medium containing these factors. Taken together, the results of this set of experiments clearly show that Bax can partially oppose the response of neurons to neurotrophic factors and that its effect on survival is dominant to that of neurotrophic factors.

The ratio of co-injected Bcl-2 and Bax expression vectors affects survival

Because Bax has been reported to inhibit the death repressor action of Bcl-2 in IL3-dependent cells (Oltvai et al., 1993), we investigated whether Bax has a similar function in neurons by co-injecting neurotrophic factor-deprived DMTG neurons with Bcl-2 and Bax expression vectors. In these experiments, the ratio between the Bcl-2 and Bax expression vectors was altered whilst keeping the combined concentration of injected vectors the same (100 μg/ml). When either expression vector was injected in excess (2:1 or 1:2, Bcl-2 vector : Bax vector) many NGF-deprived neurons were rescued compared with control injected neurons; an excess of Bcl-2 over Bax being more effective than an excess of Bax over Bcl-2 (Fig. 5). However, when the expression vectors were injected at a ratio of 1:1, The number of surviving neurons was no greater than control injected neurons.

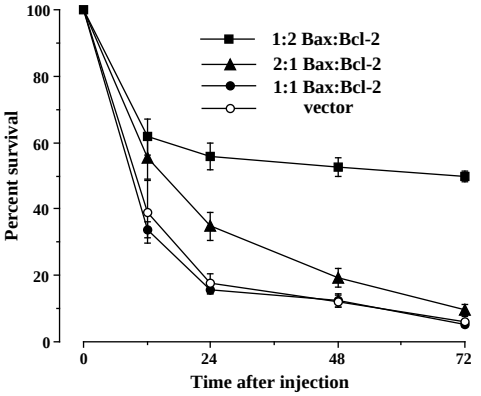

Fig. 5. Effects of co-injecting NGF-deprived DMTG neurons with Bcl-2 and Bax expression vectors. Purified cultures of E12 DMTG neurons were grown for 12 hours with 5 ng/ml NGF. After washing to remove the NGF, the neurons were co-injected with Bcl-2 and Bax expression vectors at ratios of 2:1, 1:1 and 1:2 (total DNA concentration in all cases was 100 μg/ml) or with vector without insert. The number of surviving neurons was monitored for 3 days following injection and is expressed as a percentage of the number of injected neurons. The graph shows the results of three separate experiments. In each experiment, three Petri dishes of cells were used for each condition and between 50 and 70 neurons were injected in each dish. The means and standard errors for the combined results of these experiments are shown.

DISCUSSION

We have used microinjection to introduce expression vectors synthesising members of the Bcl-2 family of intracellular proteins into cultured neurons to understand how these proteins might influence neuronal survival or modulate the survival response of neurons to neurotrophic factors. Previous microinjection studies have shown that overexpression of Bcl-2 is able to prevent the death of many sympathetic and sensory neurons deprived of members of the NGF family of neurotrophins but is ineffective in rescuing neurons that were initially grown with CNTF, GPA or bFGF (Garcia et al., 1992; Allsopp et al., 1993, 1995). Our present work has shown that overexpression of Bcl-x_L or Bcl-xβ is as effective as Bcl-2 overexpression in rescuing NGF-deprived and BDNF-deprived neurons. Like Bcl-2, neither Bcl-x_L nor Bcl-xβ are able to rescue CNTF-deprived ciliary neurons. These results suggest that Bcl-2, Bcl-x_L and Bcl-xβ have similar potency and specificity in rescuing neurons deprived of different neurotrophic factors.

The similar effect of Bcl-x_L and Bcl-xβ in preventing the death of neurotrophin-deprived neurons indicates that the C-terminal hydrophobic domain of Bcl-x is not required for its death-suppressor function in neurons. Studies of Bcl-2 suggest that its C-terminal hydrophobic domain is necessary for the attachment to the protein cytosolic side of cell membranes (Nguyen et al., 1993; Tanaka et al., 1993) but does not appear to be required for its death-suppressor function (Borner et al., 1994).

The most unexpected result of our study is that overexpression of Bax is also able to prevent the death of neurons deprived of neurotrophic factors. Not only does Bax rescue sensory neurons deprived of either NGF or BDNF, it also rescues some ciliary neurons deprived of CNTF. Although it is not quite as effective as Bcl-2, Bcl-x_L and Bcl-xβ in rescuing neurotrophin-deprived sensory neurons and its effect on CNTF-deprived ciliary neurons is transient, these survival-promoting effects of Bax are clear and statistically significant. Thus, contrary to the widely accepted view that Bax promotes cell death, based on studies of an IL-3-dependent cell line (Korsmeyer et al., 1993; Oltvai et al., 1993; Oltvai and Korsmeyer, 1994), our findings suggest that Bax overexpression does not invariably cause apoptosis, but can promote the survival of neurons following neurotrophic factor deprivation. Moreover, Bax seems to have a broader spectrum of survival-promoting activity among neurons than Bcl-2, Bcl-x_L or Bcl-xβ. However, because we studied the effects of murine Bax in chicken neurons we cannot rule out the possibility that evolutionary divergence in the Bax gene might result in murine Bax acting as a dominant negative in chicken cells.

Our study has shown that Bax overexpression is also able to inhibit the survival response of neurons to neurotrophic factors; Bax overexpression reduces the number of neurons surviving with neurotrophic factors to the number that survive when Bax is overexpressed in the absence of neurotrophic factors. In contrast, Bcl-2 overexpression does not antagonise the survival response of neurons to neurotrophic factors; the survival of Bcl-2-overexpressing neurons is slightly better in the presence of neurotrophic factors. The mechanism by which Bax promotes neuronal survival in the absence of neurotrophic factors whilst at the same time inhibiting the survival effect of these factors is unclear. Cultured neurons might express one or

more members of the Bcl-2 family of proteins that may be required for the survival response of neurons to neurotrophic factors. Sensory neurons and ciliary neurons, for example, are known to express *bcl-2* mRNA (Allsopp et al., 1995). The demonstration that antisense *bcl-2* RNA reduces the level of endogenous *bcl-2* mRNA and inhibits the survival response of sensory neurons to BDNF but does not affect the survival response of ciliary neurons to CNTF, GPA or bFGF (Allsopp et al., 1995), suggests that endogenously expressed Bcl-2 is required for the long-term survival response to BDNF but not to CNTF, GPA or bFGF. It is possible that overexpressed Bax protein may compete with Bcl-2 and/or other members of the Bcl-2 family of proteins and form heterodimers with these proteins which may interfere with their function and compromise the survival response to neurotrophic factors. Although Bax contains BH1 and BH2 domains, which are thought to be required for the survival effect of Bcl-2 family members (Yin et al., 1994), and can promote neuronal survival in the absence of neurotrophic factors, Bax is less effective in doing so than Bcl-2 or Bcl-x. Unfortunately, the small numbers of purified embryonic neurons available for our studies precludes direct measurements of the relative levels of Bcl-2-related proteins in these neurons.

In contrast to the demonstration that Bax inhibits the death repressor action of Bcl-2 in IL3-dependent cells (Oltvai et al., 1993), we have shown that co-injection of Bax and Bcl-2 expression vectors in sensory neurons does not accelerate cell death. When either expression vector was in excess (2:1 or 1:2, Bcl-2 vector : Bax vector) many NGF-deprived neurons were rescued (excess Bcl-2 vector being more effective than Bax vector). However, when the expression vectors were injected at a ratio of 1:1, survival was no greater than vector-injected controls. Although we do not know the relative levels of Bcl-2 and Bax proteins expressed in injected neurons and cannot assess this because of the very small number of neurons available for analysis, our results raise the possibility that when either Bcl-2 or Bax proteins are expressed in excess in neurotrophic factor-deprived neurons, cell death is prevented.

Site-directed mutagenesis of Gly 145 in the BH1 domain or Trp 188 in the BH2 domain of Bcl-2 prevents Bcl-2 from forming heterodimers with Bax but does not impede the formation of Bcl-2 homodimers. Because these mutations inactivate the death repressor action of Bcl-2, it has been proposed that Bcl-2 must bind to Bax in order to exert its death-repressor activity (Yin et al., 1994). However, there are alternative explanations for the effects of these mutations. First, it is possible that the Bcl-2 homodimer rather than the Bcl-2/Bax heterodimer is the active suppressor of cell death and that these mutations inactivate the death-repressor function of Bcl-2 without affecting homodimerisation. Our demonstration that overexpression of Bax prevents the death of neurotrophic factor-deprived neurons raises the possibility that Bax homodimers may also have a death-repressor function. Second, mutations in the BH domains may not only prevent the formation of Bcl-2/Bax heterodimers but prevent Bcl-2 from associating with other proteins that might be important for suppressing cell death. For example, Bcl-2 has been shown to associate with the *ras*-related protein R-*ras* p23 (Fernadez-Sarabia and Bischoff, 1993) and with a novel protein, BAG-1, that enhances the survival-promoting effect of Bcl-2 (Takayama et al., 1995).

After our study was submitted for publication, the generation of mice with a null mutation in the *bax* gene was reported (Knudson et al., 1995). These mice display both hyperplasia (increased numbers of B and T cells) and hypoplasia (testis atrophy associated with an increased numbers of apoptotic cells). These findings provide additional new evidence that, in addition to promoting cell death, Bax may promote the survival of certain cell types. Analysis of the nervous system of these mice and studies of their neurons in culture will clarify the extent to which Bax acts as a positive or negative regulator of cell survival in the nervous system.

This work was supported by a grant from the Cancer Research Campaign. G. M. is supported by a BBSRC Studentship. Our thanks to Ruth Edgar for technical assistance.

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(Accepted 22 November 1995)