Bax promotes neuronal cell death and is downregulated during the development of the nervous system

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SUMMARY

The Bcl-2 and Bcl-x proteins suppress programmed cell death, whereas Bax promotes apoptosis. We investigated the pattern of expression of Bcl-2, Bax and Bcl-x during neuronal differentiation and development. All three proteins were widely expressed in neonatal rats but, in the adult, Bax levels were 20- to 140-fold lower in the cerebral cortex, cerebellum and heart muscle, whereas Bcl-x was not downregulated in any of the tissues examined. In the cerebral cortex and cerebellum, the decrease in Bax levels occurred after the period of developmental cell death. Further, microinjection of a Bax expression vector into cultured sympathetic neurons, which depend on nerve growth factor for survival, induced apoptosis in the presence of survival factor and increased the rate of cell death after nerve growth factor withdrawal. This effect could be blocked by co-injection of an expression vector for Bcl-xL or for the baculovirus p35 protein, an inhibitor of caspases (ICE-like proteases). These results suggest that, during development, the sensitivity of neurons to signals that induce apoptosis may be regulated by modulating Bax levels and that Bax-induced death requires caspase activity.

Key words: apoptosis, Bax, Bcl-x, neuronal development, cell death, nervous system, rat

INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a form of cell death in which the cell actively participates in its own destruction and which is characterised by a specific series of morphological and biochemical changes (Wyllie et al., 1980; Ellis et al., 1991; Raff, 1992). Cell death by apoptosis is important for many aspects of normal development, for tissue homeostasis and as a defence against viral infection (Ellis et al., 1991; Raff, 1992). In the case of the vertebrate nervous system, approximately half of the neurons produced by neurogenesis die by apoptosis during embryonic and early postnatal development (Oppenheim, 1991). Neuronal apoptosis is modulated by specific survival factors, such as the neurotrophins, which are synthesized in limiting quantities by neuronal targets (Levi-Montalcini, 1987; Barde, 1989; Raff et al., 1993). Neurons are produced in excess and therefore compete for neurotrophin and those that fail to obtain adequate amounts die by apoptosis. This is thought to be a mechanism for ensuring that neuronal targets are innervated by the correct density of neurons.

We have been investigating the molecular mechanisms of programmed cell death in the developing nervous system (Rubin et al., 1994; Ham et al., 1995) and, in this study, have focused on the role of members of the bcl-2 family of genes, which encode proteins that regulate the rate at which cell populations undergo apoptosis. The bcl-2 proto-oncogene was discovered at a chromosomal translocation in human lymphoid malignancies (Tsujimoto et al., 1985; Cleary and Sklar, 1985) and encodes a 26×10^3 M_r membrane-associated protein. The Bcl-2 protein has been shown to block apoptosis induced by a variety of treatments in a number of different mammalian cell types, including myeloid cells, lymphocytes (reviewed by Nunez and Clarke, 1994; Korsmeyer, 1995) and neurons (Garcia et al., 1992; Allsopp et al., 1993), as well as developmental PCD in the nematode worm Caenorhabditis elegans (Vaux et al., 1992).

It is now clear that bcl-2 is only one member of a family of genes (Nunez and Clarke, 1994; White, 1996) and the different members of the bcl-2 family can be divided into two categories according to their effects on apoptosis. The products of one group of genes delay cell death. These proteins include Bcl-2, Bcl-xL (the long isoform of Bcl-x), adenovirus E1b 19k and the Epstein Barr Virus BHRF1 (White, 1996). A second group of genes encodes proteins that promote apoptosis. These include Bax, Bak, Bik, Bad and Bcl-xS, the short isoform of Bcl-x (White, 1996). Of these proteins, the best characterized, in addition to Bcl-2, are Bcl-x and Bax. Three different forms of Bcl-x, namely Bcl-xL, Bcl-xB and Bcl-xS, have been described and are the result of alternative mRNA splicing (Boise et al., 1993; Gonzalez-Garcia et al., 1994). Like Bcl-2, Bcl-xL and Bcl-xB delay apoptosis induced by a variety of treatments in cell lines or primary neuronal cultures whereas Bcl-xS promotes apoptosis (Boise et al., 1993; Gonzalez-Garcia et al., 1995).

Bax was the first member of the Bcl-2 family to be shown to promote apoptosis and was originally identified as a protein that interacted with Bcl-2 (Oltvai et al., 1993). Bax accelerated the rate of death of an IL-3-dependent cell line deprived of...
survival factor. Furthermore, overexpressed Bax could counteract the death repressor activity of Bcl-2 (Oltvai et al., 1993) and analysis of point mutations in Bcl-2 suggested that direct binding of Bcl-2 to Bax was essential for the death-repressor activity of Bcl-2 (Yin et al., 1994). These results led to a model in which the ratio of Bcl-2 to Bax determines whether cells survive or die following an apoptotic stimulus (Korsmeyer et al., 1993). Bax also interacts with and antagonizes Bcl-xL but mutational analysis indicated that Bcl-xL could suppress apoptosis by a mechanism that did not require binding to Bax (Cheng et al., 1996).

Since the balance between the levels of the pro- and anti-apoptotic members of the Bcl-2 family determines how particular cell types will respond to signals that induce apoptosis, studying the function and regulation of these proteins in neurons will be important for understanding the mechanisms of neuronal cell death (reviewed by Davies, 1995). Here we have used specific antibodies to study the pattern of expression of the Bax, Bcl-x and Bcl-2 proteins during neuronal differentiation and development of the nervous system. We have used the PC12 cell line as a model system for studying neuronal differentiation and found that Bax, but not Bcl-x, decreases in level when PC12 cells are induced to differentiate in vitro by treatment with nerve growth factor (NGF), suggesting that downregulation of Bax may be associated with neuronal differentiation. Consistent with these in vitro observations, we also find that Bax, but not Bcl-x, is downregulated 20- to 140-fold during the postnatal development of the cerebral cortex and cerebellum. In the case of both the cortex and cerebellum, Bax is expressed at high levels during the period when programmed cell death normally takes place and downregulation occurs after this time. To investigate whether such alterations in the ratio of Bax to Bcl-x could affect neuronal survival, we tested the effect of overexpressing Bax in developing sympathetic neurons. Microinjection of a Bax expression vector induced apoptosis even in the presence of survival factor and this cell death could be blocked by co-expression of Bcl-xL or the baculovirus p35 protein, an inhibitor of the caspase (ICE protease) family. These results demonstrate that the ratio of Bax to Bcl-x changes dramatically during the development of the nervous system and that the survival of neurons depends on a delicate balance between the level of Bax and the levels of other members of the Bcl-2 family that suppress apoptosis, such as Bcl-x. Furthermore, cell death induced by Bax requires the activity of one or more members of the caspase family since it can be effectively blocked by overexpression of the baculovirus p35 protein.

MATERIALS AND METHODS

Cell culture

PC12 cells were grown in a defined medium consisting of DMEM supplemented with high glucose supplemented with SATO mix (Doherty et al., 1988). Undifferentiated PC12 cells were cultured in defined medium supplemented with 2% foetal calf serum, 10 µg/ml insulin and 1% penicillin-streptomycin. Cells were plated at a density of 2×10⁶ in 75 cm² plastic culture flasks coated with poly-L-lysine and collagen prepared from rat tails. Stock cultures were passaged once a week and maintained at 37°C in a humidified atmosphere containing 8% CO₂. The medium was changed every 3 days. PC12 cells were differentiated by plating at a density of 1×10⁶ in 9 cm dishes in defined medium supplemented with NGF (100 ng/ml). Only early passage PC12 cells were used for differentiation experiments.

Sympathetic neurons from the superior cervical ganglia of 1-day-old Sprague-Dawley rats were isolated and cultured as described by Ham et al. (1995). The neurons were plated on glass coverslips coated with poly-L-lysine and laminin, for microinjection and immunofluorescence experiments.

NGF withdrawal experiments were carried out as follows. The medium was carefully removed from dishes of sympathetic neurons. The cells were gently rinsed with medium lacking NGF and then were refed with ~NGF medium, to which neutralising anti-NGF antibody (Boehringer) had been added.

Extract preparation and immunoblotting

Whole-cell extracts were prepared from PC12 cells as described by Ham et al. (1995). Extracts were prepared from tissues as follows: tissues were dissected from 1-, 8-, 15-, or 21-day-old and adult (3-month-old) Sprague Dawley rats and were immediately frozen and ground in liquid nitrogen, followed by lysis in 0.5 ml 2x concentrated SDS lysis buffer (Ham et al., 1995). The lysates were heated at 90°C for 30 minutes and were then centrifuged at 100,000 g for 30 minutes at 4°C in a benchtop ultracentrifuge (Beckman). The resulting supernatants were removed and stored at ~80°C prior to protein determination by the Bradford assay (Bio-Rad).

Proteins were separated on 15% SDS-polyacrylamide gels using the Bio-Rad mini Protein II electrophoresis system. 10 µg of extract was loaded per lane. After electrophoresis, the proteins were transferred onto Hybond ECL nitrocellulose membrane using a Bio-Rad mini-tranblot electrophoretic transfer cell. Immunoblotting was carried out by standard procedures (Harlow and Lane, 1988). The Bax and Bcl-x proteins were detected by using affinity-purified rabbit polyclonal antibodies (dilution 1:100). The Bax and Bcl-x antibodies were raised against peptide sequences from the amino-terminus of the murine Bax and human Bcl-x molecules. The Bax peptide was NH₂-IQDRAGRMAGETPELTLEQPPQDASTK - COOH and the Bcl-x peptide was NH₂-QFSDVNEENRTAEPGETSEMETK - COOH. The peptides were keyhole limpet haemocyanin using glutaraldehyde and were injected into rabbits subcutaneously. After several rounds of immunisation, specific antibodies were isolated from the immune sera by affinity chromatography on peptide-ε-carboxyhexanoyl (ECH)-Sepharose (Pharmacia) columns. In some immunoblotting experiments, a Bcl-x antibody from Transduction Laboratories was used. Bcl-2 was detected by using a rabbit anti-Bcl-2 mouse serum (a gift from Gerard Evan, ICRF). Primary antibodies were detected using a horseradish peroxidase-conjugated anti-rabbit secondary antibody and the ECL chemiluminescence detection system (Amersham). Signal strength was determined by scanning films with an imaging densitometer (Bio-Rad).

Preparation of tissue sections and immunohistochemistry

Brain tissue was dissected from 8-day-old and adult (3-month-old) Sprague-Dawley rats. Tissues were immediately frozen in powdered dry ice prior to sectioning. 10 µm thick sagittal sections were cut along the cortex and cerebellum using a cryostat (Bright) and sections were mounted on gelatin-coated slides. Sections were fixed in 3% paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature, and then were treated for 2 minutes with cold (~20°C) methanol. For staining with the Purkinje-specific marker calbindin, sections were fixed in 2 N HCl for 30 minutes at room temperature followed by extensive washing in PBS. Blocking was in 50% goat serum, 10% donkey serum and 1% BSA in TBST (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20). All primary antibody incubations were at room temperature for 1 hour except for the calbindin antibody, which was incubated overnight. The Bax and Bcl-x antibodies were diluted 1:50. To identify neuronal and glial populations, some P8 sections were double stained with a polyclonal antibody specific for neuron-specific...
enolase (NSE, Affinity Labs, 1:100) and a glial fibrillary acidic protein (GFAP)-specific monoclonal antibody (Boehringer, 1:100). In the case of the adult cerebellum, granule cells were identified by staining with a polyclonal antibody specific for the GABA receptor α6 subunit (1:100 dilution), a gift from Anne Stephenson (School of Pharmacy). Purkinje neurons were identified by using a monoclonal antibody specific for calbindin D-28K (Sigma, diluted 1:1000). Following incubation with primary antibody, sections were incubated for 1 hour at room temperature with a biotinylated anti-rabbit antibody (1:400, Vector Laboratories Inc.) and FITC-conjugated avidin (Vector, 1:400). In the case of sections where the primary antibody was calbindin or GFAP, an anti-mouse TRITC-conjugated secondary antibody (Jackson, 1:100 dilution) was used. Washing between incubations was in PBS containing 0.1% Tween 20. Nuclei were stained with Hoechst (0.3 µg/ml in H2O). Fluorescence staining was visualized using a Nikon Microphot FXA microscope. Photographs were taken on Kodak TMY 400 film.

**Plasmid constructions**

BclII/EcoRI fragments containing the human bax and bcl-x cDNAs were cut from plasmids provided by G. Evan and were subcloned into the CMV expression vector pcDFLAG so that the FLAG epitope was fused in frame at the amino-terminus of each open reading frame. pcDFLAG was constructed by cloning a double-stranded oligonucleotide containing the FLAG epitope (NH2-MDYKDDDDK-COOH) between the HindIII and BamHI sites of pcDNA1 (Invitrogen). The structure of the plasmids was verified by direct sequencing using the T7 and SP6 promoter primers. The construction of pCDp35 is according to M. J. McCarthy (unpublished data).

**Microinjection**

Microinjection experiments were carried out as previously described (Ham et al., 1995). The expression vectors pCDBax and pCDBcl-x were injected in 0.5x PBS, either separately or together, each at a final concentration of 0.05-0.1 mg/ml. pCDp35 was injected at a concentration of 0.8 mg/ml. When the vectors were injected separately, the DNA concentration was made up to the appropriate final concentration by adding pcDNA1. DNA was injected directly into the nucleus. In each experiment, 200 neurons were injected per injection mix. Injected cells were maintained in the presence of NGF for 24 hours prior to fixation.

**Immunofluorescence analysis of injected cells**

24 hours after injection, the cells were fixed with 3% paraformaldehyde for 30 minutes at room temperature, washed twice in PBS and permeabilized in 0.5% Triton X-100 in PBS for 5 minutes at room temperature. Blocking was with 50% goat serum in 1% BSA/PBS. Primary and secondary antibodies were diluted in 1% BSA in PBS. Incubations were for 1 hour each, at room temperature. Expression of FLAG-Bax, FLAG-Bcl-x or FLAGp35 was visualized by staining with the FLAG-specific M2 monoclonal antibody (IBI Kodak) diluted at 1:200 and an FITC-conjugated goat anti-mouse secondary antibody (Jackson), diluted 1:100. To identify injected cells, a rhodamine-conjugated anti-guinea pig IgG antibody (Jackson) was used at 1:100. Nuclei with fragmented DNA were detected by TUNEL analysis using a kit from Boehringer and nuclear morphology was visualized by Hoechst staining (10 µg/ml in water). In experiments where the effect of pCDBax, pCDBcl-x and pCDp35 on neuronal viability was quantitated, the slides were scored in a blinded fashion.

**Effect of Bax on NGF-withdrawal-induced death**

Sympathetic neurons were injected with pcDNA1, pCDBax or pCDp35. pCDBax + pCDBcl-x, as described above. As a marker, 70x103 M−1 Texas Red dextran (Molecular Probes) was included in the injection mix at a final concentration of 5 mg/ml. 3 hours after injection, the cells were rinsed once and then were refed with medium lacking NGF, to which neutralising anti-NGF antibody (Boehringer) had been added. The number of surviving injected neurons was then determined by counting the number of Texas Red-containing nuclei using an inverted fluorescence microscope. The cells were then recounted at different times after NGF withdrawal and the percentage of viable neurons was calculated. The cells were counted in a blinded fashion.

**RESULTS**

**Differential regulation of Bax and Bcl-x expression during PC12 cell differentiation**

To be able to study the pattern of expression of the Bax and Bcl-x proteins in the nervous system, we raised specific antibodies against human Bax and Bcl-x. Rabbits were immunized with peptides containing sequences from the amino terminus of each protein and the resulting antisera were affinity purified (see Materials and Methods). In immunoprecipitation experiments with in vitro translated human Bax and Bcl-x, we confirmed that the antibodies recognized the appropriate

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**Fig. 1.** Bax, but not Bcl-x, levels decrease when PC12 cells are differentiated with NGF. (A) Freshly plated, proliferating PC12 cells were refed with defined medium supplemented with either 0.1% FCS or NGF at 100 ng/ml and whole-cell extracts were prepared at the times shown. Extracts were fractionated by SDS-PAGE on 15% gels and were transferred to nitrocellulose. 15 µg of extract was loaded per lane. The Bax and Bcl-x proteins were detected with affinity-purified antibodies as described in Materials and Methods. The position and size (×10^-3 kDa) of molecular mass markers that were run on the same gels are shown on the right. (B) The films shown in (A) were scanned on a densitometer and the amounts of Bax and Bcl-x were determined. For each timepoint, the amount of Bax was divided by the amount of Bcl-x. The amount of Bax divided by the amount of Bcl-x at 0 hours was set at 100 and the values at other times were calculated in relation to this and plotted against time after the addition of medium containing NGF or 0.1% FCS.
proteins. The Bcl-x antiserum recognized both Bcl-xL and Bcl-xS translated in vitro. We also showed in immunoblotting experiments with extracts from cell lines that the antibodies also recognized the rat and mouse Bax and Bcl-x. In these experiments, the specificity of the antibodies was confirmed by peptide competition (data not shown).

To study the pattern of expression of Bax and Bcl-x during neuronal differentiation, we used the rat PC12 cell line as a model system. When treated with NGF, PC12 cells initially complete the cell cycle to which they are committed, then stop dividing and acquire a neuronal phenotype similar to that of developing sympathetic neurons (Greene and Tischler, 1976). Like developing sympathetic neurons, differentiated PC12 cells also depend on NGF for survival. In initial experiments, we found that PC12 cells expressed Bax and Bcl-xL, but not Bcl-xS or Bcl-2 protein (data not shown). We then investigated whether the levels of Bax and Bcl-x were affected by NGF treatment.

Proliferating PC12 cells were initially plated in defined medium containing 2% foetal calf serum (FCS) and insulin. The next day, the cells were refed with medium lacking serum and insulin but supplemented with NGF. Control cells were plated in medium supplemented only with 0.1% FCS. Whole-cell extracts were prepared at different times after changing the medium and immunoblots were carried out using the Bax and Bcl-x antibodies. As shown in Fig. 1A, NGF treatment over 72 hours caused a gradual but steady reduction in the level of Bax protein. At the latest time point (72 hours), Bax protein levels had dropped to less than 10% of their original level (Fig. 1B). The level of Bax protein remained constant in the control extracts (medium + 0.1% FCS) indicating that the decrease in the level of Bax was due to the addition of NGF and not to the removal of serum and insulin from the medium. In contrast to Bax, the level of Bcl-x protein was not affected by NGF addition, indicating that Bax and Bcl-x are differentially regulated during PC12 differentiation.

To confirm that the downregulation of Bax protein in PC12 cells treated with NGF was related to neuronal differentiation, we also studied the effect of the cyclic AMP analogue CPTcAMP, an agent which, like NGF, can induce PC12 differentiation and which will also promote the survival of differentiated PC12 cells and developing sympathetic neurons. Treatment with 100 µM CPTcAMP also caused a decrease in Bax protein levels and appeared to work as well as NGF (Fig. 2A,B). The two agents did not appear to have an additive effect (Fig. 2). As before, Bax levels did not decrease significantly when the level of serum in the medium was reduced. In contrast to Bax, Bcl-x levels were not changed by these treatments.

**Fig. 2.** CPTcAMP also causes Bax downregulation in PC12 cells. (A) Extracts were prepared from PC12 cells grown for 72 hours in defined medium containing different concentrations of FCS or 100 ng/ml NGF or 100 µM CPTcAMP or NGF in combination with CPTcAMP. Extracts were fractionated on 15% SDS-polyacrylamide gels and the separated proteins were transferred to nitrocellulose. 15 µg of extract was loaded per lane. Immunoblots were carried out using the Bax and Bcl-x antibodies. The position and size (×10^{-3}) of molecular mass markers that were run on the same gels are shown. (B) The levels of Bax and Bcl-x were determined by scanning the films shown in (A) on a densitometer. The level of Bax or Bcl-x in extracts from cells cultured in medium containing 2% FCS was set at 100 and the other values were adjusted to this.

**Fig. 3.** Bax is downregulated during the postnatal development of the cerebral cortex, cerebellum and heart muscle in the rat. Whole-cell extracts were prepared from different tissues dissected from neonatal (P1) or 3-month-old adult (A) rats. 15 µg aliquots of each extract were fractionated on 15% SDS-polyacrylamide gels and the separated proteins were transferred to nitrocellulose. The blots were sequentially probed with the affinity purified Bax and Bcl-x antibodies as well as an anti-Bcl-2 immune serum. Molecular mass markers are shown (×10^{-3} M).
cell line in vitro. If this also occurred in primary neurons, then Bax levels would be predicted to decrease during the development of the nervous system as developing neurons exit from the cell cycle and undergo terminal differentiation. To investigate whether Bax and Bcl-x levels are differentially regulated during development, we prepared extracts from a variety of tissues dissected from 1-day-old and adult Sprague Dawley rats. The extracts were fractionated by SDS-PAGE, and immunoblots were carried out using the Bax and Bcl-x antibodies as well as an anti-Bcl-2 immune serum (Fig. 3). Bax protein levels decreased dramatically during the postnatal development of the cerebral cortex, cerebellum and heart but remained more or less constant in thymus, liver, lung, kidney and spleen. A similar pattern of expression was observed for Bcl-2, except that the Bcl-2 protein was not detected in the liver, as previously described (Hockenbery et al., 1991; Veis Novack and Korsmeyer, 1994). In contrast to Bax and Bcl-2, the level of Bcl-x protein remained unchanged in all tissues examined at both stages of development. A protein of the size of Bcl-x was not detected.

A considerable amount of neuronal cell death by apoptosis occurs during the development of the cerebral cortex and cerebellum (Wood et al., 1993; Blaschke et al., 1996). For example, in the cerebellum, granule cells undergo apoptosis in the first weeks after birth with extensive DNA fragmentation occurring between days 5 and 9 (Wood et al., 1993). To determine whether Bax was downregulated before or after this period of cell death we carried out immunoblots with extracts prepared from 1-, 8-, 15- and 21-day-old animals as well as from a 3-month-old adult rat (Fig. 4A). In the cerebral cortex, Bax levels were highest at P1, slightly reduced at P8 and P15 but substantially decreased at P21 and 3 months (Fig. 4B). The level of Bax protein in the adult cortex was 143-fold lower than that in the P1 cortex. Bcl-2 protein levels also decreased during the postnatal development of the cortex, but only by 7.5-fold. Bcl-x levels remained unchanged. In the case of the cerebellum, Bax levels were high at P1 and P8 and somewhat reduced (50% lower) at P15. Therefore Bax was present at high levels at the time in development when DNA fragmentation and programmed cell death are at a peak. From P15 onwards, Bax levels fell so that they were very low in the adult at 3 months, a time when it has been reported that relatively little apoptosis is observed in the cerebellum (Wood et al., 1993) and which we confirmed by carrying out TUNEL analysis on cerebellar sections (K. Vekrellis, unpublished observations). Bcl-2 also decreased in level during the postnatal development of the cerebellum although the decrease (almost 3-fold) was not as great as that observed for Bax (a 22-fold decrease in level). Bcl-x levels remained constant.

Our immunoblotting experiments demonstrated that the level of Bax protein decreased during the postnatal development of the cortex and cerebellum. To determine in which cell populations this occurred, we used the Bax and Bcl-x antibodies to stain sections of the brain. Frozen, adjacent sagittal sections of 8-day-old and adult rat brains were stained for Bax and Bcl-x as described in Materials and Methods. Results for the cerebellum are shown in Fig. 5. At day 8 after birth, both the immature Purkinje cells and the cells of the external germinal layer, as well as the internal granule cell layer stained strongly for both Bax and Bcl-x (Fig. 5A). Both antibodies predominantly stained the cytoplasm and the specificity of this staining was confirmed by peptide competition (data not shown). To identify which cell types were expressing Bax and Bcl-x at P8, we stained sections with antibodies specific for glial fibrillary acidic protein (GFAP) or neuron-specific enolase (NSE), which detect glial cells and neurons respectively. Both the Bax and Bcl-x antibodies gave staining

![Fig. 4](image-url)
patterns similar to NSE but different to the GFAP pattern suggesting that Bax and Bcl-x were either localised in all cells in the P8 cerebellum, or only in neurons. In the adult cerebellum, the Purkinje and granule cells also stained strongly for Bcl-x (Fig. 5B). However, when stained for Bax, granule cells in the adult tissue showed a dramatic reduction in immunofluorescence signal compared to the day 8 sections, whereas the Purkinje cells continued to express Bax at high levels (Fig. 5B). The identity of granule cells and Purkinje neurons in the adult cerebellum was established by staining with antibodies that recognize a granule cell-specific marker (the GABA<sub>A</sub> receptor α6 subunit; Thompson and Stephenson, 1994) or with an antibody against a Purkinje cell-specific marker (calbindin D-28K; Mason et al., 1990).

In conclusion, our immunohistochemical experiments demonstrated that Bax protein is expressed at high levels on day 8 of postnatal development, when developmental programmed cell death is occurring. Furthermore, the Bax staining pattern was similar to that obtained with an NSE antibody suggesting that Bax was localized in neurons and their precursors. In contrast, in the adult cerebellum where less programmed cell death occurs, Bax is expressed at low levels in the granule neurons but is still present at high levels in the Purkinje neurons.

**Overexpression of Bax in developing sympathetic neurons induces apoptosis which is blocked by co-expression of Bcl-xL or the baculovirus p35 protein**

Overexpression of Bax protein in lymphoid cell lines has pre-
Fig. 6. Overexpression of Bax in developing sympathetic neurons induces apoptosis, which can be prevented by co-expression of Bcl-xL or the p35 inhibitor of ICE proteases. (A) Sympathetic neurons were isolated from newborn rats and were cultured for 7 days in vitro in the presence of NGF. The cells were then microinjected with pCDBax or with pCDBax together with pCDBcl-xL each at a final DNA concentration of 0.1 mg/ml. The injected cells were maintained in NGF-containing medium for 24 hours and then were fixed and stained as follows: expression of Bax or Bcl-x L was visualised by staining with the FLAG-specific M2 monoclonal antibody, nuclei where DNA fragmentation had occurred were detected by TUNEL analysis and nuclear morphology was visualised by Hoechst staining. Representative injected cells are shown. The majority of cells overexpressing Bax had a typical apoptotic morphology, including a reduced cytoplasmic and nuclear volume, fragmented neurites, Hoechst positive nuclei and condensed chromatin revealed by Hoechst staining (upper three panels). In contrast, cells injected with equal amounts of pCDBax and pCDBcl-xL had a normal morphology and were not stained by the TUNEL reagent (lower three panels). The bar represents 25 μm. (B) Sympathetic neurons were microinjected with pCDBcl-xL or pCDBax or with pCDBax + pCDBcl-xL or pCDBax + pCDp35. pCDBax and pCDBcl-xL were added to the injection mixes at a final concentration of 0.05 mg/ml. pCDp35 was added to a final concentration of 0.8 mg/ml. Where necessary the empty expression vector pcDNA1 was used to adjust the final DNA concentration to 0.85 mg/ml. The injected cells were maintained in NGF-containing medium for 24 hours and then were fixed and stained as described in A. For each injection mix, the percentage of surviving injected cells stained by the M2 monoclonal antibody, i.e. those cells that expressed Bax, Bcl-x or p35, that had pyknotic nuclei was determined. The slides were scored in a blinded fashion. The results shown are the average of three independent experiments ± the standard error. In each experiment, 200 neurons were injected per injection mix.

Previously been shown to accelerate cell death induced by survival factor withdrawal and Bax expression will counteract the effect of the anti-apoptotic Bcl-2 protein (Olvanvi et al., 1993). It has therefore been suggested that the balance between the level of Bax and the levels of anti-apoptotic proteins such as Bcl-2 and Bcl-xL is critical for determining a cell's sensitivity to signals that induce apoptosis (Korsmeyer et al., 1993). To determine whether this was true in the mammalian nervous system, we investigated the effect of overexpressing Bax in sympathetic neurons isolated from neonatal rats. We selected this type of primary neuron for functional studies because sympathetic neurons have a similar phenotype to differentiated PC12 cells and because they are a well-characterized model for developmental programmed cell death. Developing sympathetic neurons depend on NGF for survival and have previously been shown to express Bax, Bcl-x and Bcl-2 (Krajewski et al., 1994; Merry et al., 1994; Greenlund et al., 1995). Furthermore, it has been demonstrated that overexpression of Bcl-2 or Bcl-xL will protect these neurons against NGF-withdrawal-induced death (Garcia et al., 1992; Gonzalez-Garcia et al., 1995). We subcloned the human Bax and Bcl-xL cDNAs into the CMV expression vector pCDFLAG so that the FLAG epitope was fused in frame at the amino terminus of each protein. The expression vectors were directly injected into the nuclei of sympathetic neurons and the expression of Bax and Bcl-xL was visualised by staining with the FLAG-specific M2 monoclonal antibody. Both FLAGBax and FLAGBcl-xL were efficiently expressed and localised to the cytoplasm of the injected cells. To determine the effect of these proteins on neuronal viability, we microinjected the expression vectors either alone or together into sympathetic neurons. The injected cells were maintained in NGF-containing medium. 24 hours after injection, the cells were fixed and treated as follows: expression of Bcl-x or Bax was visualized by staining with the FLAG-specific M2 monoclonal antibody, nuclei where DNA fragmentation had occurred were detected by TUNEL analysis and nuclear morphology was visualised by Hoechst staining. Representative injected cells are shown in Fig. 6A. Overexpression of Bax in sympathetic neurons induced apoptosis even though the injected cells had been maintained in the presence of their neurotrophic factor NGF (Fig. 6A,B). At the timepoint examined (24 hours after injection), 85% of the nuclei that had been injected with pCDBax were pyknotic as judged by Hoechst staining and most of these were labelled by the TUNEL reagent. Only about 10% of the cells injected with pCDBcl-xL exhibited apoptotic features (Fig. 6B). A similar percentage of apoptotic cells was observed when the empty expression vector, pcDNA1, was injected alone (data not shown). The death produced by overexpression of Bax in sympathetic neurons could be suppressed when an equimolar amount of pCDBcl-xL was co-injected with pCDBax (Fig. 6B). Cells injected with both vectors demonstrated reduced TUNEL staining and had morphologically normal nuclei (Fig. 6A). We also investigated whether overexpression of Bax accelerated
the NGF-withdrawal-induced death of sympathetic neurons (Fig. 7). Sympathetic neurons were microinjected with the empty expression vector pcDNA1, as a negative control, or with pCDBax or with pCDBax together with an equal amount of pCDBcl-xL. After injection, the cells were rinsed and refed with medium lacking NGF, which had been supplemented with neutralising anti-NGF antibody. The number of surviving injected neurons was then determined at different times after NGF withdrawal. Overexpression of Bax significantly increased the rate of cell death after NGF withdrawal and this effect was prevented by co-expression of Bcl-xL (Fig. 7).

The molecular mechanism by which Bax promotes apoptosis in neurons or other cell types is unknown. Recently it has been demonstrated that members of the caspase family of proteases (ICE-like proteases; Alnemri et al., 1996) are essential for neuronal cell death induced by survival factor withdrawal (Gagliardini et al., 1994; Milligan et al., 1995). We therefore investigated whether the DNA fragmentation and cell death induced by microinjection of pCDBax required the activity of members of the caspase family. To determine whether caspase activity was required, we tested the effect of co-injecting an expression vector for the baculovirus p35 protein (pCDp35) together with pCDBax. The p35 protein has been shown to directly inhibit caspase activity in vitro (Xue and Horvitz, 1995; Bump et al., 1995) and will block apoptosis in a wide variety of biological systems including sympathetic neurons deprived of NGF (Martinou et al., 1995). pCDp35 encodes a FLAG-tagged p35 protein which is efficiently expressed when the vector is microinjected into sympathetic neurons and which localizes to the cytoplasm and nucleus (M. J. McCarthy et al., unpublished data). When pCDp35 was co-injected with pCDBax, the percentage of cells with pyknotic nuclei was reduced from 85% to 14% (Fig. 6B) suggesting that cell death caused by overexpression of Bax required the activity of members of the caspase family.

**DISCUSSION**

In this study, we used antibodies specific for Bax, Bcl-x and Bcl-2 to study the pattern of expression of these proteins during neuronal differentiation in vitro and during the postnatal development of the nervous system in vivo. As a model for neuronal differentiation, we used the PC12 cell line and found that agents, such as NGF and CPTcAMP, that promote cell cycle exit, neurite outgrowth and neuronal survival significantly decreased Bax protein levels but did not alter the level of Bcl-xL. This reduction appears to be related to differentiation since, under conditions of reduced proliferation, the level of Bax protein did not decrease. It has previously been suggested that the ratio of Bax to Bcl-2 and Bcl-xL is important for regulating cell survival (Oltvai et al., 1993). Although differentiated PC12 cells resemble postmitotic sympathetic neurons, it is clear that this system does not model all aspects of the differentiation of sympathetic neurons. Nevertheless, the PC12 cell line has been a valuable model system for establishing basic mechanisms of neuronal signal transduction. The results of our experiments with NGF-treated PC12 cells suggested that, in primary neurons, the ratio of Bax to Bcl-xL might decrease markedly during differentiation, which in turn might make the differentiated cells less sensitive to signals that induce cell death. These results suggested a model in which Bax levels would be high in the early stages of neuronal development, when programmed cell death occurs, but would decrease as neurons mature and make appropriate connections with their trophic targets. To test this hypothesis, we carried out immunoblotting experiments with extracts prepared from a variety of tissues isolated from neonatal and adult rats. We found that Bax levels decreased significantly during the postnatal development of the cerebral cortex, cerebellum and heart muscle, whereas Bcl-xL levels remained constant throughout the course of development. The latter observation is consistent with the results of Gonzalez-Garcia et al. (1995), who found that Bcl-xL protein levels were similar in the embryonic and adult mouse cerebral cortex and cerebellum. It is of note that we observed Bax downregulation in tissues that consist of long-lived, post-mitotic cell types (neurons and muscle). In the case of the cerebral cortex and cerebellum, it has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax protein did not decrease. It has previously been suggested that the level of Bax
cerebellum, as previously observed by Merry et al. (1994) although, in the case of both tissues, the decrease in the level of Bcl-2 was smaller than the decrease in the level of Bax (Fig. 4B).

As well as carrying out immunoblots, we used our Bax and Bcl-x antibodies in immunohistochemistry experiments with brain sections prepared from 8-day-old and adult rats. Consistent with the immunoblotting results, Bax staining was, in general, greatly reduced in the adult cerebral cortex and cerebellum as compared with the same tissues at postnatal day 8. In contrast, the Bcl-x signal was unaltered between P8 and adult sections. We also found a few subpopulations of neurons that continued to express relatively high levels of Bax protein in the adult rat. For example, the Purkinje cells of the cerebellum contained high levels of Bax (Fig. 5B) as did the large pyramidal neurons of the CA1 region of the hippocampus (K. Vekrellis, unpublished observations). This particular pattern of expression of Bax in the adult rat nervous system was also observed by Krajewski et al. (1995), who noted that these neuronal subpopulations are highly sensitive to death induced by ischaemia, hypoglycaemia and excitatory neurotransmitters, consistent with the hypothesis that cells with a high Bax to Bcl-x ratio may be more sensitive to stimuli that induce apoptosis.

The mechanism by which NGF treatment or developmental signals cause a specific reduction in Bax protein levels is unknown at present. One possibility is that these signals might lead to a decrease in the rate of transcription of the bax gene. For example, a transcription factor that activates the bax promoter might decrease in level during postnatal development. One possible candidate is the p53 tumour suppressor protein, which can bind to the bax promoter and activate transcription of bax (Miyashita and Reed, 1995) and, in adult p53−/− knockout mice, it has been observed that Bax protein levels are decreased in the few neuronal populations that normally express Bax in the adult, such as sympathetic neurons, cerebellar Purkinje neurons and neurons in the olfactory cortex (Miyashita et al., 1994). Furthermore, it has previously been shown that p53 RNA levels decrease during the postnatal development of the rat cerebellum (Hayes et al., 1991) with a time course similar to the decrease in Bax protein levels that we have observed.

To determine whether altering the Bax/Bcl-x ratio would have an effect on differentiated, postmitotic neurons, we microinjected rat sympathetic neurons with expression vectors for the human Bax and Bcl-xL proteins. Interestingly, we found that overexpression of Bax was sufficient to cause the death of sympathetic neurons in the presence of their trophic factor NGF. In contrast, overexpression of Bcl-x only caused a small decrease in viability and this was probably a non-specific effect due to the injection procedure. Furthermore, as might be predicted, we found that overexpression of Bax accelerated the NGF-withdrawal-induced death of sympathetic neurons, as had previously been shown for the related Bak protein (Farrow et al., 1995). This is in complete contrast to the results of Middleton et al. (1996), who found that injection of a Bax expression vector into embryonic chick sensory and ciliary neurons delayed the survival factor-withdrawal-induced death of these cells, when compared with an empty expression vector. The reasons for this discrepancy are unclear. However, the same authors found that injection of a Bax expression vector into chick sensory and ciliary neurons in the presence of survival factor decreased cell viability. Thus, taken together with our data these results indicate that, in the presence of survival factor, several different types of primary neuron are sensitive to changes in the ratio of Bax/Bcl-x and suggest that pathological changes that cause Bax levels to increase above a certain threshold could potentially induce neuronal cell death. In this regard, it is interesting that Krajewski et al. (1995) observed increased levels of Bax protein in certain populations of neurons degenerating after ischaemia in the rat brain.

In our microinjection experiments, death induced by injecting pCDBax could be prevented by co-injecting an equimolar amount of a Bcl-x expression vector. Our microinjection data therefore support the hypothesis that the balance between Bax and the other anti-apoptotic members of the Bcl-2 family is critical for determining the sensitivity of cells to death stimuli. Recently the genes encoding Bcl-2, Bax and Bcl-x were disrupted in mice by homologous recombination. In bcl-2−/− mice, the nervous system was not dramatically altered (Veis et al., 1993; Nakayama et al., 1993) although degeneration of motor, sensory and sympathetic neurons occurred after the period of physiological cell death (Michaeldis et al., 1996). In contrast, extensive neuronal cell death by apoptosis occurred during the development of bcl-x−/− mice suggesting that the Bcl-x protein plays a critical role in the development of the nervous system (Motoyama et al., 1995). If Bax protein levels are as high during embryonic development as we have found them to be in neonatal animals, then the neuronal cell death observed in bcl-x−/− embryos may be the result of an excessively high Bax/Bcl-x ratio, which could trigger apoptosis as described here for developing sympathetic neurons. While this manuscript was in preparation, Deckwerth et al. (1996) reported that there were increased numbers of neurons in the superior cervical ganglia and facial nuclei of bax−/− knockout mice. Furthermore, neonatal sympathetic neurons isolated from bax−/− mice could survive NGF deprivation in vitro. These results suggest that Bax is required for neuronal death by trophic factor deprivation and during development. Taken together with the results of our microinjection experiments, these observations suggest that the level of Bax in sympathetic neurons is an important factor affecting neuronal survival.

Recent studies with stably transfected cell lines have suggested that Bcl-2 and Bcl-x may act upstream of the caspase family of proteases in the cell death pathway (Chinnaiyan et al., 1996). Consistent with this model, we found that Bax-induced apoptosis could be blocked by overexpression of the baculovirus p35 protein, an inhibitor of caspases. Furthermore, we have found that 100 μM z-VAD.fmk (benzoyloxy carbonyl-Val-Ala-Asp [OMe] fluoromethylketone), a specific peptide inhibitor of caspases (Slee et al., 1996), will also provide substantial protection against Bax-induced apoptosis (data not shown). It is not known whether Bax promotes apoptosis by preventing Bcl-xL from suppressing the cell death programme or whether the reverse is true - that Bcl-xL blocks cell death by preventing Bax from activating caspases. Although Bax and Bcl-xL have been shown to interact (Yang et al., 1995), it was recently reported that Bcl-xL does not need to bind to Bax to suppress cell death (Cheng et al., 1996). Perhaps Bcl-x prevents Bax from inducing cell death by sequestering a critical downstream target of Bax. Further experiments will be necessary to test such models.

In conclusion, our results suggest that, during development,
the sensitivity of neurons to signals that induce apoptosis may be regulated by modulating Bax levels. Furthermore, in microinjection experiments, we found that increasing the level of Bax in differentiated, postmitotic neurons can induce apoptosis even in the presence of survival factor. This result suggests that increases in the level of Bax protein that occur in certain populations of neurons degenerating after ischemia in the rat brain (Krajewski et al., 1995) could actually be the cause of the observed neurodegeneration and suggest that specific inhibitors of Bax might be of therapeutic value. In the future, it will be interesting to determine whether Bax is required for all forms of neuronal cell death, like the caspases, or whether it only plays a role in certain neuronal populations.

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