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bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death

(apoptosis/*bcl-2*/neuronal survival)

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ABSTRACT **Previous studies have implicated the *bcl-2* protooncogene as a potential regulator of neuronal survival. However, mice lacking functional *bcl-2* exhibited normal development and maintenance of the central nervous system (CNS). Since *bcl-2* appears dispensable for neuronal survival, we have examined the expression and function of *bcl-x*, another member of the *bcl-2* family of death regulatory genes. Bcl-2 is expressed in neuronal tissues during embryonic development but is down-regulated in the adult CNS. In contrast, Bcl-x_L expression is retained in neurons of the adult CNS. Two different forms of *bcl-x* mRNA and their corresponding products, Bcl-x_L and Bcl-x_β, were expressed in embryonic and adult neurons of the CNS. Microinjection of *bcl-x_L* and *bcl-x_β* cDNAs into primary sympathetic neurons inhibited their death induced by nerve growth factor withdrawal. Thus, Bcl-x proteins appear to play an important role in the regulation of neuronal survival in the adult CNS.**

During embryogenesis in vertebrates, a large fraction of the developing neurons die by programed cell death (PCD) (1, 2). The mechanism by which PCD is induced appears to occur through competition for trophic factors produced by target tissues and may serve to select for the proper set of neuronal synapses (2, 3). Once established, the great majority of postmitotic neurons in the adult animal are long-lived. The survival of neurons is thought to depend on neurotrophic factors (3, 4). In the absence of such factors, the neurons die by PCD (2–5). Previous studies have implicated the *bcl-2* protooncogene as a potential regulator of neuronal survival. First, overexpression of *bcl-2* prevented the death of primary neurons and neuronal cell lines following trophic factor deprivation (6–9). Second, Bcl-2 is expressed in a large population of neurons during embryonic development (10, 11) and in some cells in the adult brain (12). However, although the Bcl-2 protein is retained in neurons of the peripheral nervous system, Bcl-2 levels are greatly reduced or undetectable in the majority of postmitotic central nervous system (CNS) neurons of adult mice, rhesus monkeys, and humans (13). Perhaps the strongest evidence against a critical role of Bcl-2 in neuronal survival is the finding that adult mice in which the *bcl-2* gene has been disrupted by homologous recombination exhibited normal development and maintenance of the CNS (14, 15). Since *bcl-2* appears to be dispensable for the maintenance of neurons in the CNS, we hypothesized that genes other than *bcl-2* may control the survival of CNS neurons during adult life. In the present study, the expression and function of *bcl-x*, a *bcl-2*-related gene (16), were investigated in neuronal tissues and in isolated populations of neurons.

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MATERIALS AND METHODS

S1 Nuclease Assay. Murine *bcl-x* and *bcl-2* probes were labeled and hybridized simultaneously with total mRNA samples as described (17). Autoradiographs were quantified by densitometry scanning with a radioanalytic imaging system (AMBIS Systems).

***In Situ* Hybridization.** Frozen tissues were sectioned and processed as described (18). Antisense and sense RNA uridine 5′-[α-[^{35}S]thio]triphosphate-labeled probes were generated using linearized templates containing a 740-bp murine *bcl-x_L* cDNA cloned into pBluescript (17). Tissue sections were incubated with labeled probes at 55°C for 16–20 hr, digested with RNase A (1 mg/ml) at 37°C for 30 min, and washed in 50% formamide/2× SSC/0.1 M dithiothreitol at 65°C for 30 min, 2× SSC, and 0.1× SSC (two times each) for 15 min at room temperature (18). Sections were developed with Kodak NTB2 nuclear emulsion solution and visualized under dark-field microscopy.

Western Blot Analysis. Western blot analysis was performed as described (19) except that the final reaction was developed with the ECL substrate (Amersham). Each lane was loaded with 80 μg of protein and duplicate filters were incubated with 2A1.2, a monoclonal antibody against Bcl-x proteins (L.H.B. and C.B.T., unpublished data), or 3F11, which recognizes murine Bcl-2 (19).

Microinjection of DNA into Neurons. Construction of plasmids to express a FLAG tag attached to Bcl-x proteins has been described (17). Primary cultures of rat sympathetic neurons were prepared from superior cervical ganglia of newborn rats as described (20). Sympathetic neurons were maintained for 7 days in the presence of 0.1 μg of nerve growth factor (NGF) per ml and cytosine arabinonucleoside C (10 μM) to avoid growth of nonneuronal cells. Individual neurons were injected in the nucleus (DNA solution at 100 ng/ml) with a mechanical Leitz manipulator (6). The percentage of microinjected neurons expressing FLAG-Bcl-x_L or FLAG-Bcl-x_β was determined at 20 hr following injection using M2, a monoclonal antibody specific for FLAG (International Biotechnologies), followed by rhodamine-conjugated rat anti-mouse IgG (Boehringer Mannheim).

RESULTS

Two *bcl-x* mRNA Forms Are Expressed in Embryonic and Adult Neuronal Tissues. To begin to analyze *bcl-x* in neuronal tissues of mice, a quantitative S1 nuclease assay was developed to assess different forms of *bcl-x* mRNA (Fig. 1*A*). mRNA species were simultaneously hybridized with end-labeled *bcl-2* and *bcl-x* probes to allow assessment of the relative abundance

Abbreviations: CNS, central nervous system; PCD, programed cell death; NGF, nerve growth factor.

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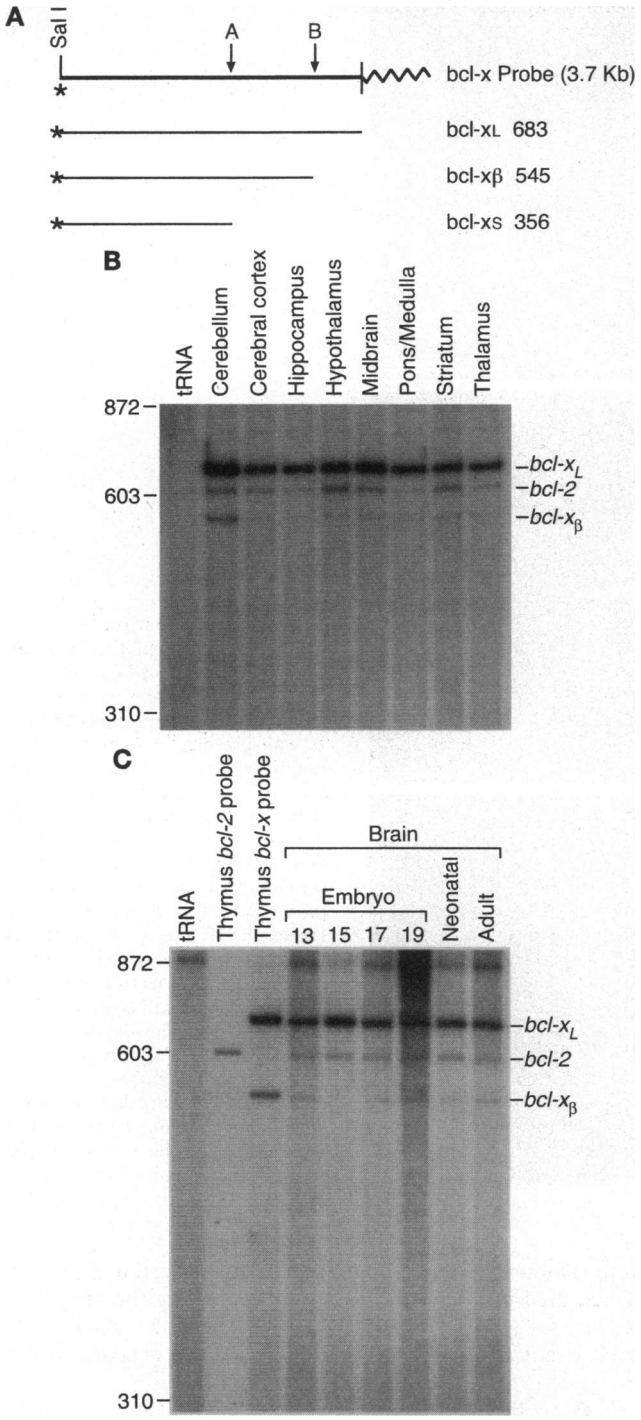

FIG. 1. Expression of *bcl-2* and *bcl-x* mRNAs in embryonic and adult neuronal tissues of mice. (*A*) S1 nuclease probe constructed to detect *bcl-x* mRNA forms. The region corresponding to the 5′ splice donor site used in the generation of *bcl-x*$_S$ mRNA in the human (16) is indicated by the A arrow. The exon/intron boundary in the *bcl-x*$_L$ cDNA is indicated by the B arrow. pBluescript sequences are indicated by a wavy line. Expected sizes of protected fragments for *bcl-x*$_L$, *bcl-x*$_S$, and *bcl-x*$_\beta$ mRNA forms are indicated in nucleotides. (*B* and *C*) Equal amounts of end-labeled *bcl-2* and *bcl-x* S1 nuclease probes were simultaneously hybridized to 10 μg of total RNA from adult tissues (*B*) or 5 μg of RNA from 13- to 19-day embryonic and neonatal tissues. (*C*) Hybridization of both probes with a control tRNA sample is shown for comparison. Hybridization of thymus mRNA to the *bcl-2* or *bcl-x* probe alone is also shown. The *bcl-2* probe protected a fragment of 600 nucleotides (17).

of *bcl-2* and *bcl-x* mRNAs. Results shown in Fig. 1 revealed that *bcl-x*$_L$ mRNA is the major species of *bcl-x* expressed in embryonic and postnatal neuronal tissues. Similar amounts of *bcl-x*$_L$ were present in several regions of the adult brain, including cerebellum, cerebral cortex, hippocampus, hypothalamus, midbrain, pons/medulla, striatum, and thalamus (Fig. 1*B*). In addition to *bcl-x*$_L$, another species of *bcl-x*, *bcl-x*$_\beta$, was expressed during development and in the adult CNS (Fig. 1*B*). Murine *bcl-x*$_\beta$ results from an unspliced *bcl-x* mRNA transcript and encodes a protein of 209 amino acids (17). The levels of *bcl-x*$_L$ mRNA in the brain were higher (5- to 55-fold) than those of *bcl-x*$_\beta$ at day 13 through day 19 of embryonic development as well as in newborn mice and in all sites evaluated in the adult brain (Fig. 1 *B* and *C*). *bcl-2* was found to be expressed in all CNS samples analyzed, which confirms results from previous reports (10–12).

Detection of *bcl-x* in CNS Neurons by *in Situ* Hybridization. We examined various neuronal tissues by *in situ* hybridization to determine the localization of *bcl-x* mRNA within individual neurons. Fig. 2 shows that *bcl-x* is expressed in neurons of the cerebral cortex, hippocampus (granular layer), and cerebellum (internal granule cell layer and Purkinje cells) from adult mice. In the 13-day embryo, *bcl-x* was present in the brain and in the ventral portion of the forming spinal cord. Some expression was also observed in the olfactory epithelium at this stage of development (Fig. 2*A*).

Differential Regulation of Bcl-2 and Bcl-x Proteins in the CNS. In agreement with the mRNA analysis, the Bcl-x$_L$ protein was detected in embryonic brain and in the cerebral cortex, cerebellum, basal ganglia, and spinal cord of adult mice (Fig. 3*A*). The expression of the Bcl-x$_\beta$ protein in the CNS was greatly reduced or undetectable when compared to that of Bcl-x$_L$, which is consistent with the mRNA analysis (Fig. 3*A*). In a control lane, the expression of Bcl-x$_L$ or Bcl-x$_\beta$ was essentially undetectable in adult liver, which is in agreement with the pattern of expression of *bcl-x* mRNA in that tissue (17). In contrast to Bcl-x proteins, Bcl-2 was detected in embryonic cortex and cerebellum but was greatly diminished or undetectable in adult CNS tissues (Fig. 3*B*). Thus Bcl-2 and Bcl-x proteins are differentially regulated in the CNS.

***bcl-x*$_L$ and *bcl-x*$_\beta$ Inhibit Neuronal Cell Death Induced by NGF Withdrawal.** To assess the ability of *bcl-x* to regulate neuronal cell death, we constructed expression plasmids for microinjection into primary neurons (Fig. 4*A*). About 80–90% of the injected neurons survived the stress of microinjection and in the presence of NGF, about 85% survived through day 5. Twenty hours after injection, >80% of the neurons that received DNA containing FLAG-*bcl-x*$_L$ or FLAG-*bcl-x*$_\beta$ were stained with anti-FLAG monoclonal antibody (Fig. 4*B*). Within 3 days of NGF deprivation, >90% of the control neurons (injected with vector alone or antisense FLAG-*bcl-x*$_L$ plasmid) died, with <5% of the cells surviving to day 5 (Fig. 4*C*). In contrast, 45% ± 4% and 43% ± 4% of the neurons injected with FLAG-*bcl-x*$_L$ survived through day 3 and day 5, respectively, in the absence of NGF (Fig. 4*C*). Interestingly, 40% ± 4% and 37% ± 5% of the neurons injected with the FLAG-*bcl-x*$_\beta$ construct survived after NGF withdrawal (Fig. 4*C*). Injection of FLAG-*bcl-x*$_S$, which encodes a protein with an internal 63-amino acid deletion in Bcl-x$_L$, failed to inhibit neuronal cell death (Fig. 4*C*).

Coexpression of Bcl-x$_L$ and Bcl-x$_\beta$ Has an Enhanced Effect on Neuronal Survival. Because Bcl-x$_L$ and Bcl-x$_\beta$ contain unique sequences, we hypothesized that their ability to inhibit neuronal cell death could be additive. To test this possibility, neurons were injected with *bcl-x*$_L$ and *bcl-x*$_\beta$ expression vectors. The survival of the coinjected neurons was nearly additive when compared to neurons injected with Bcl-x$_L$ or Bcl-x$_\beta$ expression plasmids alone at day 3 (74% ± 3% vs. 45% ± 4% or 40% ± 4%; $P = 0.001$) and day 5 (64% ± 5% vs. 43% ± 4% or 37% ± 5%; $P = 0.001$) (Fig. 4*C*). The enhanced effect of combined Bcl-x$_L$ and Bcl-x$_\beta$ on neuronal survival was not due to increased dose of the

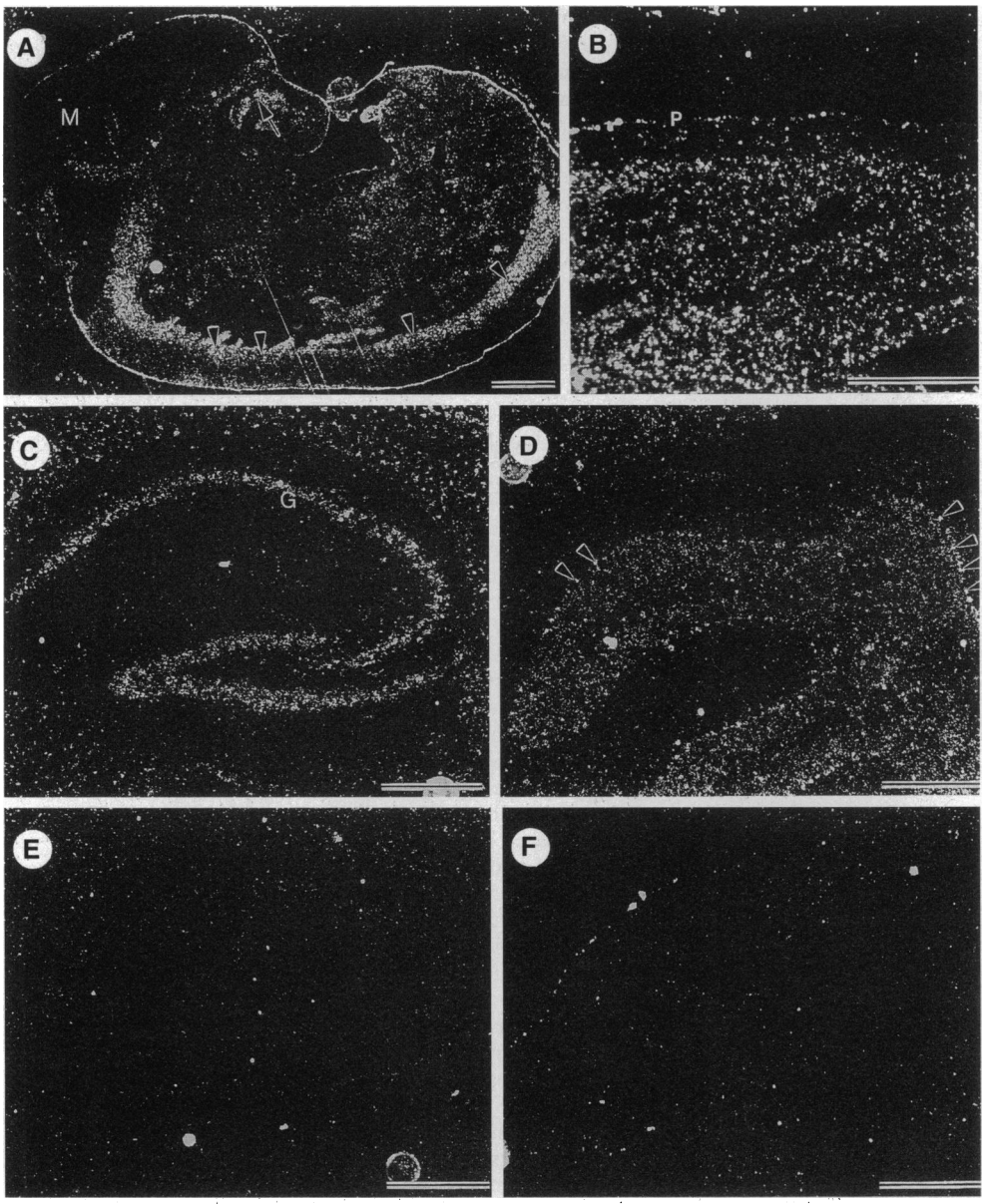

FIG. 2. Detection of *bcl-x* mRNA in neuronal tissues by *in situ* hybridization. (*A*) In the embryo at day 13, *bcl-x* was expressed in the developing nervous system. It was present in the brain (M, midbrain) and in the ventral portion of the forming spinal cord (arrowheads). Some expression was also observed in the olfactory epithelium (arrow) at this stage of development. (*B–F*) In the adult brain, *Bcl-x* was expressed in the cortex (*B*) (P, pial surface). It was expressed in the granular layer (G) of the hippocampus (*C*) and in the internal granule cell layer and Purkinje cells (arrowheads) of the cerebellar cortex (*D*). There was a nonpatterned scatter of silver grains in sections of hippocampus (*E*) and cerebellum (*F*) exposed to sense-strand probes. [Bars = 500 μm (*A*) and 50 μm (*B–F*).]

introduced plasmids since the total amount of plasmid injected was identical in the singly and doubly injected neurons. Furthermore, delivery of increasing amounts of *bcl-x*$_L$ or *bcl-x*$_\beta$ plasmids alone did not increase the survival of the injected neurons indicating that the concentration of each plasmid injected was saturating (I.G. and G.N., data not shown).

DISCUSSION

Initial studies suggested that *bcl-2* was an important regulator of neuronal survival in the CNS by repressing the apoptotic mechanism (6–9). However, recent studies have shown that Bcl-2 is not detected in populations of CNS neurons in the

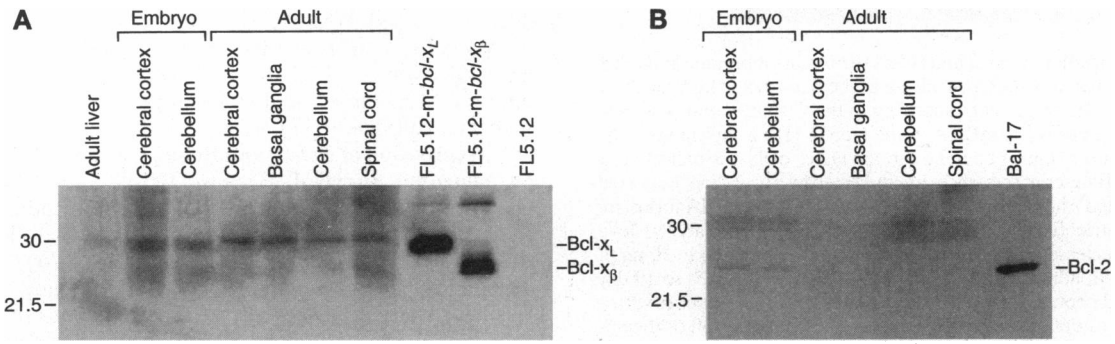

FIG. 3. Detection of Bcl-x and Bcl-2 proteins in embryonic and adult CNS tissues. (*A*) Western blot using anti-Bcl-x antibody. Cellular lysates (80 μg per lane) were obtained from an embryo at day 17 of development or a 20-week-old mouse. Lysates from parental FL5.12 cells or stably transfected with the murine *bcl-x*$_L$ (FL5.12-m-*bcl-x*$_L$) or *bcl-x*$_\beta$ (FL5.12-m-*bcl-x*$_\beta$) cDNAs are shown as controls. (*B*) Western blot using anti-Bcl-2 antibody. Lysate from BAL-17, a murine B-cell lymphoma cell line, is shown as a positive control. Size markers are in kilodaltons.

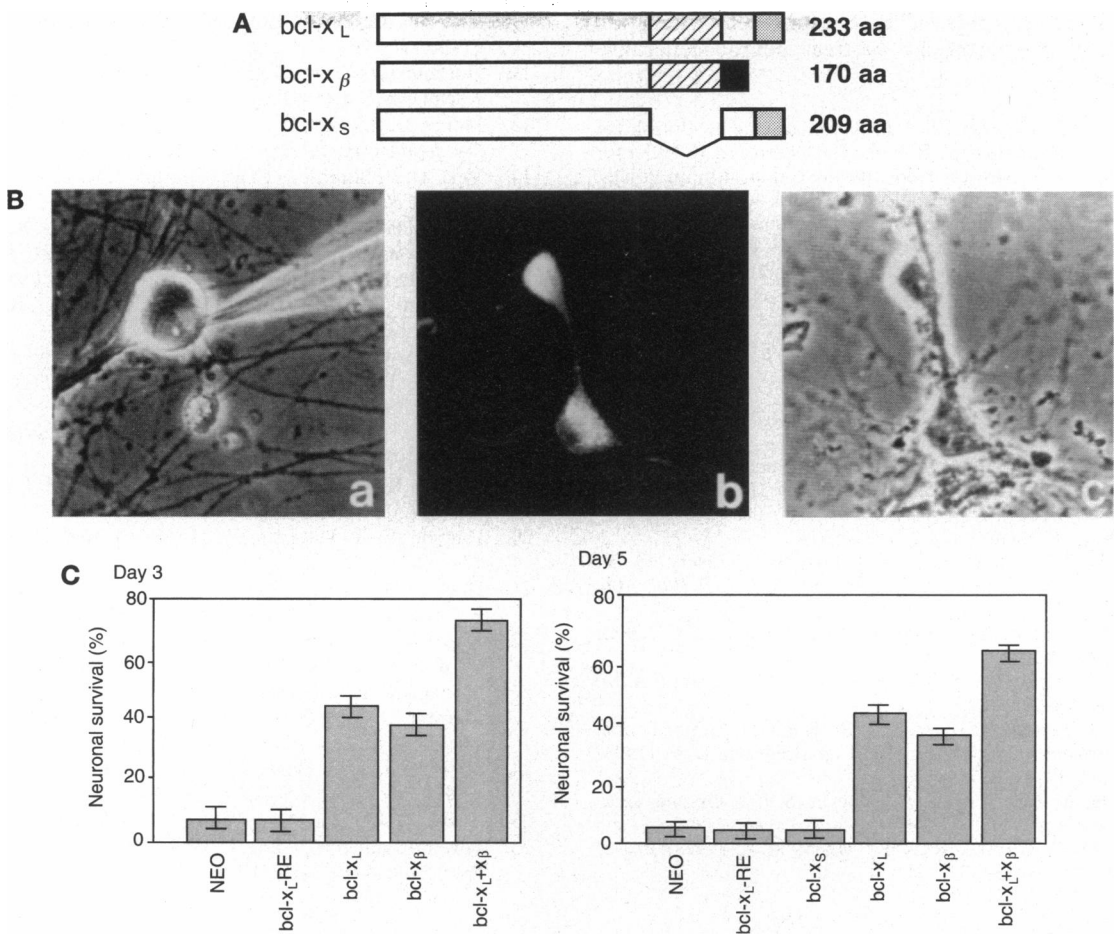

FIG. 4. Microinjection of *bcl-x*$_L$ or *bcl-x*$_\beta$ cDNAs prevents death of sympathetic neurons deprived of NGF. (*A*) Diagram of the coding sequence of *bcl-x* cDNAs. The hatched bar represents the region of high amino acid homology between Bcl-2 and Bcl-x proteins (16, 17). The dotted bar represents the hydrophobic domain at the C terminus of Bcl-x$_L$ and Bcl-x$_S$. The black bar represents a 21-amino acid stretch unique to Bcl-x$_\beta$ (17). *bcl-x* cDNAs were inserted into the pSFFV plasmid (17). (*B*) Neurons were cultured for 7 days in medium containing NGF before injection with plasmid DNA. (*a*) Plasmids were microinjected into the nucleus of individual neurons. (×190.) (*b* and *c*) Fluorescence (*b*) and phase-contrast (*c*) images of neurons injected with pSFFV-bcl-x$_L$ and labeled 20 hr later with M2, a mouse monoclonal antibody against FLAG. Greater than 80% of the neurons microinjected were positively stained. (×80.) (*C*) Survival of microinjected neurons following NGF withdrawal. Two hours after injection, the neurons were washed and cultured in NGF-free medium. Three and 5 days after injection, the cells were stained with the vital marker acridine orange and positive cells were determined as percentage of total neurons present compared to those counted 2 hr after microinjection. The results are shown as the mean percentage of surviving neurons ± SEM observed in three to five experiments (at least 100 cells microinjected for each experiment). Differences between *bcl-x*$_\beta$ or *bcl-x*$_L$ and *bcl-x*$_L$+*bcl-x*$_\beta$ were significant ($P = 0.001$, Student's *t* test).

adult (13). Our studies demonstrate that Bcl-x$_L$, a Bcl-2 homologue, is expressed in embryonic and adult neurons in the CNS. Bcl-2 and Bcl-x$_L$ exhibit a differential developmental switch in neurons of the CNS. Bcl-2 and Bcl-x proteins are expressed during embryonic development. However, although Bcl-x$_L$ is retained, the expression of Bcl-2 is greatly diminished or undetectable in populations of neurons in the adult CNS (ref. 13; Fig. 3*B*). The finding of residual *bcl-2* mRNA in the adult CNS may reflect *bcl-2* expression mainly by microglial cells as these cells have been shown to express Bcl-2 by immunohistochemistry (13). Thus, based on the levels of expression of both proteins, Bcl-x appears to be physiologically more relevant than Bcl-2 in the maintenance of neuronal viability in the adult CNS.

Our studies indicate that Bcl-x$_L$ is active against neuronal cell death and may regulate neuronal survival *in vivo*. In addition, we have shown that Bcl-x$_\beta$, another *bcl-x* gene product, can protect neurons from cell death. The additive effect of Bcl-x$_L$ and Bcl-x$_\beta$ in inhibiting neuronal cell death suggests that, at least in part, both proteins counter cell death by a different mechanism or, alternatively, by functioning at different cellular sites. Because Bcl-x$_\beta$ contains a unique stretch of 21 amino acids at the C terminus and lacks 19 hydrophobic amino acids present in Bcl-x$_L$ (17), it may display a subcellular localization different to that of Bcl-x$_L$ and/or interact with different regulatory targets.

Recent studies have suggested that Bcl-2 inhibits cell death by acting in an antioxidant pathway (8, 21). Given the high level of structural homology between Bcl-2 and Bcl-x proteins, it is likely that both proteins prevent cell death by a similar mechanism. Furthermore, Bcl-2 and Bcl-x$_L$ localize to outer mitochondria and nuclear envelope (17, 22, 23), suggesting that both proteins share a similar mechanism of action. Of interest, however, is the recent finding that Bcl-x$_L$ appears more efficient than Bcl-2 in protecting certain lymphoid cell lines from PCD induced by interleukin 3 deprivation (16) or chemotherapy drugs (24). Thus, it is possible that CNS neurons, by producing Bcl-x$_L$ rather than Bcl-2, can be protected more effectively than lymphocytes from death-inducing stimuli. Several neurodegenerative diseases are characterized by an abnormal rate of neuronal cell death and the mechanism of cellular demise has been shown to be apoptotic (25, 26). Thus, the expression of Bcl-x proteins in neurons may provide a potential approach to counter diseases characterized by pathological forms of neuronal cell death. In this regard, delivery of *bcl-x* gene constructs or identification of soluble factors or

drugs capable of up-regulating Bcl-x expression or function may provide a novel strategy to treat neurodegenerative diseases.

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