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Bcl-x_S can form homodimers and heterodimers and its apoptotic activity requires localization of Bcl-x_S to the mitochondria and its BH3 and loop domains

L Lindenboim[1], C Borner[2] and R Stein*,[1]

[1] Department of Neurobiochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Ramat Aviv, Israel
[2] Institute for Molecular Medicine and Cell Research, Zentrale Klinische Forschung (ZKF), Albert-Ludwigs-University of Freiburg, Breisacherstrasse 66 D-79106 Freiburg, Germany
* Corresponding author: R Stein, Department of Neurobiochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Ramat Aviv, Israel. Tel: 972-3-6408608; Fax: 972-3-6407643; E-mail: reuvens@POST.TAU.AC.IL

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Abstract

Proteins of the Bcl-2 family regulate apoptosis, some antagonizing cell death and others, such as Bcl-x_S, promoting it. We previously showed that expression of Bcl-x_S in PC12 cells is a useful system for studying the mechanism of Bcl-x_S-induced apoptosis. To further investigate this apoptotic effect and its prevention by anti-apoptotic agents, we assessed the role of distinct Bcl-x_S domains, via the study of their mutations, on the ability of Bcl-x_S to induce apoptosis and to localize to the mitochondria, as well as the ability of these domains to counteract the effects of anti-apoptotic agents on Bcl-x_S. Deletion of the transmembrane domain (ΔTM) prevented the localization of Bcl-x_S ΔTM to the mitochondria and the ability of this mutant to induce apoptosis. Deletion of the amino acids GD 94–95 from the BH3 domain, or deletion of the loop region, impaired the ability of these mutants to induce apoptosis but not their localization to the mitochondria. Deletion of the BH4 domain or destruction of the caspase cleavage site in the loop region (by replacing amino acid D61 with A61) did not affect either the localization of these mutants to the mitochondria or their ability to induce cell death. It thus appears that Bcl-x_S-induced apoptosis in PC12 cells is mediated by localization of Bcl-x_S to the mitochondria by a process that requires the transmembrane domain. Furthermore, once localized to the mitochondria Bcl-x_S requires the BH3 domain, and to a lesser extent the loop domain, for its subsequent activity. The anti-apoptotic agents Bcl-2 and Bcl-x_L, the caspase inhibitor Z-VAD-FMK, and nerve growth factor (NGF) did not prevent Bcl-x_S localization to the mitochondria, and did not require the BH4 or the loop domains of Bcl-x_S for their survival effect. Bcl-x_S is capable of forming homodimers with itself and heterodimers with Bcl-x_L or Bcl-2. Accordingly co-expression of Bcl-x_S ΔTM with Bcl-x_S, Bcl-2, or Bcl-x_L leads to a change in the subcellular distribution of Bcl-x_S ΔTM, from a diffuse distribution throughout the cell to a more defined distribution. Moreover co-immunoprecipitation experiments directly demonstrated that Bcl-x_S can associate with GFP-Bcl-x_S, Bcl-x_L, or Bcl-2. These results suggest that such Bcl-x_S interactions may be important for the mechanism of action of this protein. *Cell Death and Differentiation* (2001) **8,** 933–942.

Keywords: Bcl-x_S; mutations; dimerization; mitochondria; apoptosis; PC12 cells

Abbreviations: NGF, nerve growth factor; PBS, phosphate-buffered saline; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone; TM, transmembrane; SEAP, secreted alkaline phosphatase; WT, wild-type; PCR, polymerase chain reaction; TBS, Tris-buffered saline

Introduction

Programmed cell death (apoptosis) plays an important role in a wide variety of physiological processes, as well as in pathological cellular insults.[1,2] Two groups of proteins have been shown to play an important role in apoptosis: (i) a family of cysteine proteases, the caspases, which cleave substrates after aspartic residues;[3,4] and (ii) the Bcl-2 family, which contains promoters of death (such as Bax, Bak, Bok, Bad, Bik, Bid, Bim, HrK, BIK, and Bcl-x_S) and inhibitors of death (such as Bcl-2, Bcl-x_L, Bcl-W, Mcl-1, A-1, adenovirus E1B 19K, Epstein-Barr virus BHRF1, and *Caenorhabditis elegans* CED-9).[5–7] The homology between members of the bcl-2 family of proteins resides mainly within four conserved domains, the Bcl-2 homology (BH) domains, designated BH1, BH2, BH3, and BH4, which correspond to α-helical segments.

The BH1 and BH2 domains are found in all death antagonists of the Bcl-2 family, such as Bcl-2, Bcl-x_L, and Mcl-1, but in death agonists, only in those from the Bax subfamily (Bax, Bak, Bok). They are essential for the survival function of the death suppressors and for interactions of these proteins with death agonists such as Bax and Bak.[8,9] The ability of members of the Bcl-2 family of proteins to form homo- as well as heterodimers has led to the suggestion that their mechanism of action involves neutralizing competition with themselves.[10,11]

The BH3 domain is found in all the proteins of the Bcl-2 family. In the pro-apoptotic members it is needed for their death-promoting activity and for heterodimerizing with the anti-apoptotic members. For example, the BH3 domain of Bak has been shown to form an amphipathic α-helix that binds with high affinity to a hydrophobic pocket created by the BH1, BH2, and BH3 domains of Bcl-x_L.[12]

The BH4 domain occurs in most of the anti-apoptotic members of the family but only in one pro-apoptotic member, Bcl-x_S.[13] This domain is probably involved in protein–protein interactions with regulatory proteins other than those of the Bcl-2 family, such as the protein kinase Raf-1,[14] the protein phosphatase calcineurin,[15] and the mammalian homolog of the nematode caspase activator CED-4.[16]

The three-dimensional structure of Bcl-x_L contains an unstructured, flexible loop that lies between the BH4 and the BH3 domains.[17] This region is believed to serve as a site of negative regulation, but is not essential for this protein's anti-apoptotic function.[18] The loop regulatory sites include, for example, (i) phosphorylation sites within the region that can modulate the survival function of the protein,[19] and (ii) a caspase cleavage site, at Asp 61 of Bcl-x_L. Cleavage of this site was shown to convert anti-apoptotic Bcl-x_L to a pro-apoptotic protein.[20]

Most proteins in the Bcl-2 family also harbor C-terminal signal-anchor sequences (also termed the transmembrane [TM] domain) that is believed to be responsible for targeting them predominantly to the outer mitochondrial membrane, as well as to the endoplasmic reticular membrane and the outer nuclear envelope.[21] Deletion of the TM domain suppresses or even abolishes their function in some systems,[21,22] but has no effect in others.[23] It was also shown that as a result of apoptotic stimuli some pro-apoptotic proteins (e.g. Bax and Bid) translocate to the mitochondria, where they induce mitochondrial damage that results in turn in the execution of apoptosis.[7,24]

The *bcl-x* gene has three alternative splice forms, bcl-$x_{L(\alpha)}$, bcl-$x_{(\beta)}$, and bcl-$x_{S(\gamma)}$.[13,25] The Bcl-x_L splice form encodes a 233 amino acid protein containing the four BH domains and the loop and transmembrane regions. Bcl-x_S, however, as a result of alternative splicing, lacks an internal 63 amino acid segment that contains the conserved BH1 and BH2 domains. It therefore contains, in addition to the loop and transmembrane regions, only the BH3 and BH4 domains. This unique structure of Bcl-x_S assigns it to a unique position among the other pro-apoptotic members of the Bcl-2 family, as it is the only member which contains the BH4 domain. Moreover, Bcl-x_S differs from the Bax-like members (which also contain a loop region) in that it lacks the BH1 and BH2 domains.

Accumulating evidence suggests that Bcl-x_S may act as a pro-apoptotic protein in various apoptotic systems. For example, Bcl-x_S mRNA was shown to be increased in several apoptotic systems,[26,27] and the expression of Bcl-x_S cDNA was found to induce apoptosis in various cancer cells.[28–30] We recently showed that overexpression of Bcl-x_S leads to its mitochondrial localization and to caspase-dependent apoptosis in PC12 cells,[31] a well-characterized cellular model system commonly used for the study of neuronal apoptosis.[32,33] In an attempt to better understand how the unique structure of Bcl-x_S affects its pro-apoptotic action, we examined the role of distinct Bcl-x_S domains in apoptosis induced by overexpression of Bcl-x_S in PC12 cells. Several Bcl-x_S mutants were generated in different domains and regions. Our results showed that the transmembrane region, the BH3 domain, and – to a lesser extent – the loop region are required for apoptosis induced by Bcl-x_S in these cells. In addition, examination of the subcellular localization of the different Bcl-x_S mutants revealed that the localization of Bcl-x_S to the mitochondria, an event mediated by the TM domain, is an early and important prerequisite of its apoptotic effect. After Bcl-x_S has been targeted to the mitochondria, its BH3 domain and to a lesser extent the loop region are required for continuation of the apoptotic process. We also examined the interaction of Bcl-x_S with itself and with the anti-apoptotic proteins Bcl-x_L and Bcl-2. The results show that Bcl-x_S is capable of forming homodimers with itself and heterodimers with Bcl-x_L and Bcl-2, suggesting that such interactions may be important for its mechanism of action.

Results

Identification of Bcl-x_S domains required for cell death

We recently established a model system for studying the mechanism of Bcl-x_S-induced apoptosis by overexpression of Bcl-x_S in PC12 cells.[31] In an attempt to identify the Bcl-x_S regions that are important for this apoptotic effect, we generated a series of mutations in different domains of Bcl-x_S and tested them for their ability to induce apoptosis in PC12 cells. Using the previously described structure of Bcl-x_L as a reference,[17] we generated the following Bcl-x_S mutants as N-terminal FLAG tag fusion proteins (Figure 1): Bcl-x_S ΔBH4 (BH4 domain deleted); Bcl-x_S Δloop (loop region deleted and replaced by three alanines); Bcl-x_S ΔGD (the two well-

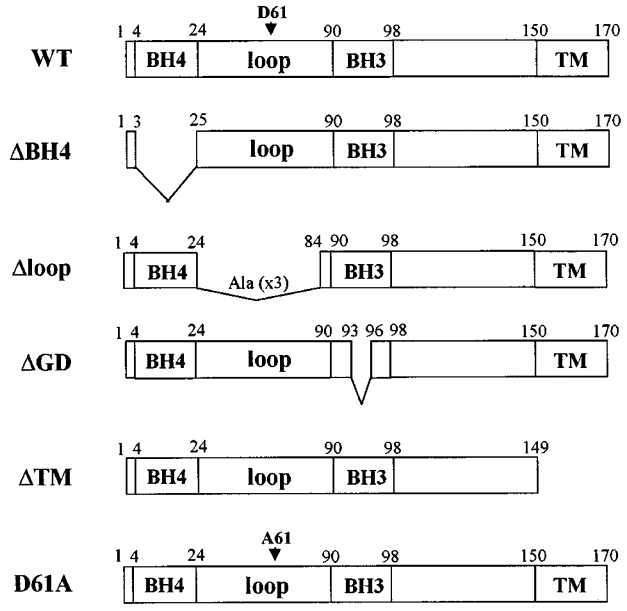

Figure 1 Structures of Bcl-x_S mutants. The structures of the Bcl-x_S WT and Bcl-x_S mutants [Bcl-x_S ΔBH4 (ΔBH4), Bcl-x_S Δloop (Δloop), Bcl-x_S ΔGD (ΔGD), Bcl-x_S ΔTM (ΔTM), and Bcl-x_S D61A (D61A)] are illustrated schematically. Numbers refer to the amino acids of Bcl-x_S wild-type protein. All constructs were generated as described in Materials and Methods, and all contain an amino-terminal FLAG epitope tag

conserved amino acids, glycine and aspartic acid, deleted from the BH3 domain); Bcl-x_S ΔTM (the C-terminal hydrophobic domain, also termed the transmembrane domain, deleted), and Bcl-x_S D61A (the caspase cleavage site in the loop region destroyed by conversion of the aspartic acid at position 61 to alanine).

PC12 cells were transiently co-transfected with the expression vector of a Bcl-x_S mutant and the expression vector for secreted alkaline phosphatase (SEAP) reporter gene, and the effect of the expression of each Bcl-x_S mutant on cell viability was determined by monitoring SEAP activity in the transfected cultures after 24 h. As shown in Figure 2A, transfection of PC12 cells with Bcl-x_S expression vector induced a dose-dependent reduction in SEAP activity in the transfected cells. Deletion of the BH4 domain, or mutation of the caspase cleavage site in the loop region, had no effect on Bcl-x_S-induced cell death, as shown by the ability of Bcl-x_S ΔBH4 and Bcl-x_S D61A to reduce the viability of the transfected cells as much as or more than in Bcl-x_S wild-type (WT). On the other hand, deletion of the transmembrane domain or the amino acids GD from the BH3 domain completely (in the case of Bcl-x_S ΔTM) or almost completely (80% survival in the case of 0.65 μg/ml Bcl-x_S ΔGD) abolished the ability of Bcl-x_S to induce apoptosis. Deletion of the loop region (Bcl-x_S Δloop) partially interfered with the ability of Bcl-x_S to induce apoptosis. Accordingly, cell death was not observed when the cells were transfected with low concentrations of Bcl-x_S Δloop plasmid, whereas at higher concentrations, where Bcl-x_S WT induced 82% cell death, Bcl-x_S Δloop induced cell death of only 59%. These results, which were obtained by measuring SEAP activity as an indicator of cell viability, were confirmed by evaluating the number and morphology of GFP-positive cells in each experiment (data not shown). Examination of the expression levels of the different transfected Bcl-x_S proteins by Western blot analysis (Figure 2B) revealed that they were all roughly equivalent, suggesting that the different effects of these proteins on cell viability are not due to differences in their expression levels but rather a result of differences in their properties. Taken together, the findings suggest that Bcl-x_S requires its transmembrane domain, its BH3 domain, and – to a lesser extent – its loop domain for its cell-killing effect in PC12 cells.

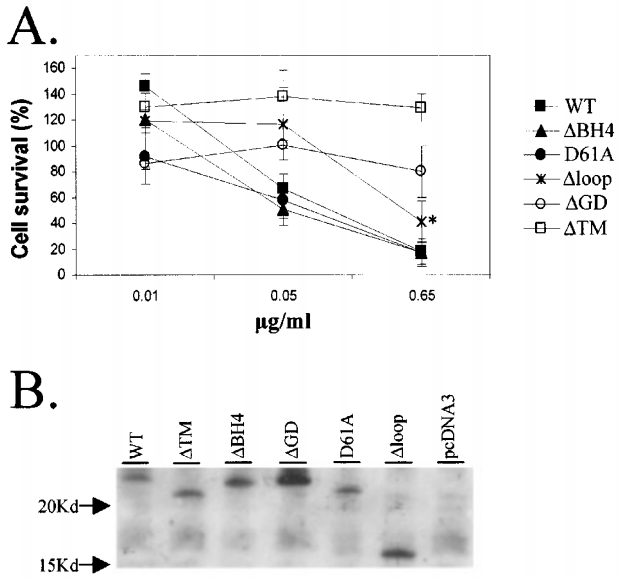

Figure 2 Identification of the Bcl-x_S domains required for Bcl-x_S-induced cell death. PC12 cells were transiently co-transfected for 24 h with the reporter plasmid SEAP and the indicated concentrations of Bcl-x_S WT or mutants (see Figure 1 for description of mutants). SEAP activity (**A**) in each transfection was determined after 24 h, as described in Materials and Methods. Cell survival is defined as SEAP activity in cultures transfected with Bcl-x_s WT or mutants as a percentage of SEAP activity in cultures transfected with the control vector pcDNA3. Data are expressed as mean values ± S.D. (n=3 for 0.01 and 0.05 μg/ml DNA; n=4 for 0.65 μg/ml DNA). *Denotes a significant difference in the percentage of cell survival between Δloop and Bcl-x_S WT ($P<0.02$, $t_{(3)}=4.9$), as assessed by paired t-test. The expression level (**B**) of each of the different Bcl-x_S forms was determined in PC12 cultures transfected with 0.65 μg/ml of plasmid DNA. Cells were lysed and 100 μg of protein from each transfection was subjected to SDS–PAGE (12.5%) immunoblot analysis using anti-Flag antibody, as described in Materials and Methods. The data shown are from a representative experiment whose SEAP values were included in the analysis of the data presented in **A**

The transmembrane domain of Bcl-x_S is required for the mitochondrial localization of Bcl-x_S

We have previously shown that exogenous overexpressed Bcl-x_S protein is localized to the mitochondria in PC12 cells, suggesting that the localization of Bcl-x_S to the mitochondria is important for its death effect.[31] To verify this assumption, we examined the ability of the Bcl-x_S mutants to localize to the mitochondria and sought to correlate the mitochondrial localization of these mtuants with their ability to induce apoptosis.

PC12 cells were transiently transfected with the FLAG-tagged Bcl-x_S WT or the expression vectors of the Bcl-x_S mutants, and their subcellular localization was determined 24 h later by staining the cells with anti-FLAG antibody. As shown in Figure 3, immunofluorescence analysis of the subcellular localization of the various Bcl-x_S proteins by confocal microscopy showed that Bcl-x_S ΔBH4, Bcl-x_S

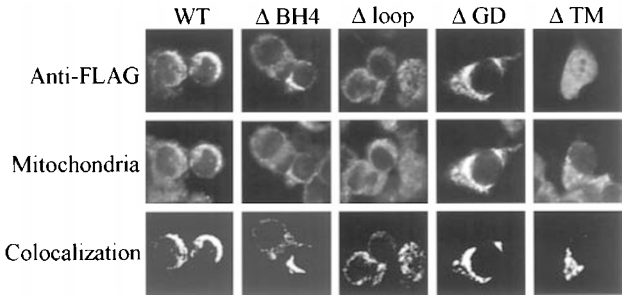

Figure 3 The transmembrane domain of Bcl-x_S is required for its mitochondrial localization. PC12 cells were transiently transfected with either FLAG-tagged Bcl-x_S WT or mutant (see Figure 1 for description of mutants) expression vectors. After 24 h the cells were treated with 250 nM MitoTracker Red, fixed, permeabilized, and incubated with monoclonal mouse anti-FLAG antibody and then with fluorescein isothiocyanate-conjugated second antibody. Cells were imaged by 2-color confocal immunofluorescence microscopy (×4800 magnification). Co-localization of Bcl-x_S WT or mutants with the mitochondria is revealed by overlaying of the images

Δloop, and Bcl-x_S ΔGD, as well as Bcl-x_S WT, exhibited a punctuated immunoreactivity in the cells consistent with an association with the mitochondria (as indicated by double staining with anti-FLAG antibody and the mitochondria-specific dye, MitoTracker Red), whereas Bcl-x_S ΔTM was expressed throughout the cell (except for the nucleolus) and was not localized to any organelle-like structure. The results depicted in Figures 2 and 3 thus suggest that in PC12 cells the localization of Bcl-x_S to the mitochondria is a necessary but insufficient event for its apoptotic effect. After being localized to the mitochondria, Bcl-x_S requires its BH3 domain and – to a lesser extent – the loop region for the apoptosis to proceed.

Bcl-2, Bcl-x_L, Z-VAD-FMK, and NGF do not prevent localization of Bcl-x_S to the mitochondria

We have previously shown that co-expression of Bcl-x_S with Bcl-2 or Bcl-x_L, or treatment with the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone (Z-VAD-FMK) or the survival factor nerve growth factor (NGF), can inhibit cell death induced by Bcl-x_S in the cells.[31] The finding that prevention of Bcl-x_S localization to the mitochondria (by removal of the TM domain) inhibits its apoptotic effect may suggest that the mechanism whereby Bcl-2, Bcl-x_L, NGF, and Z-VAD-FMK suppress Bcl-x_S-induced apoptosis involves such prevention. We therefore attempted to determine whether these survival agents would inhibit the localization of Bcl-x_S to the mitochondria.

PC12 cells were transiently co-transfected with FLAG-tagged Bcl-x_S WT and either Bcl-2 or Bcl-x_L expression vectors, or with FLAG-tagged Bcl-x_S WT expression vector in the presence of NGF (50 ng/ml) or Z-VAD-FMK (100 μM). The subcellular localization of Bcl-x_S was determined 24 h later by staining the cells with anti-FLAG antibody. As shown in Figure 4, confocal immunofluorescence microscopy analysis showed that neither co-expression of Bcl-2 or Bcl-x_L with Bcl-x_S nor treatment with NGF or Z-VAD-FMK prevented the appearance of Bcl-x_S in the mitochondria, as indicated by the fact that the immunoreactivity pattern of Bcl-x_S was consistent with its mitochondrial association. These results thus suggest that the mechanism by which Bcl-2, Bcl-x_L, NGF, and Z-VAD-FMK inhibit Bcl-x_S-induced apoptosis in PC12 cells is not mediated by preventing the localization of Bcl-x_S to the mitochondria.

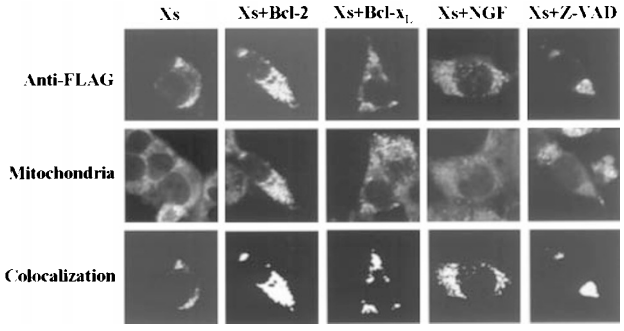

Figure 4 Bcl-2, Bcl-x_L, Z-VAD-FMK, and NGF do not prevent localization of Bcl-x_S to the mitochondria. PC12 cells were transiently co-transfected with FLAG-tagged Bcl-x_S WT (X_S) alone or with Bcl-2 or Bcl-x_L expression vectors, or in the presence of 50 ng/ml NGF or 100 μM Z-VAD-FMK. After 24 h the cells were treated with 250 nM MitoTracker Red and processed as described for Figure 3

The BH4 and the loop domains of Bcl-x_S are not required for the survival effects of Bcl-x_L, Bcl-2, NGF, and Z-VAD-FMK

To better understand the mechanism by which the anti-apoptotic molecules, Bcl-2 and Bcl-x_L, and the agents NGF and Z-VAD-FMK prevent Bcl-x_S-induced cell death, we examined whether the BH4 domain or the loop region of Bcl-x_S is required for the survival effects of Bcl-2, Bcl-x_L, NGF, and Z-VAD-FMK. PC12 cells were co-transfected with Bcl-x_S WT or Bcl-x_S ΔBH4 or Bcl-x_S Δloop and SEAP reporter gene, and either co-transfected with Bcl-x_L or Bcl-2 expression vectors or treated with 50 ng/ml NGF or 100 μM Z-VAD-FMK. The effect of each treatment on the viability of the transfected cells 24 h later was examined by monitoring SEAP activity. As shown in Figure 5, Bcl-2 and Bcl-x_L, as well as NGF and Z-VAD-FMK, protected PC12 cells from the apoptotic effect of Bcl-x_S ΔBH4 or Bcl-x_S Δloop to the same extent as they protected them from the apoptotic effect of Bcl-x_S WT. These

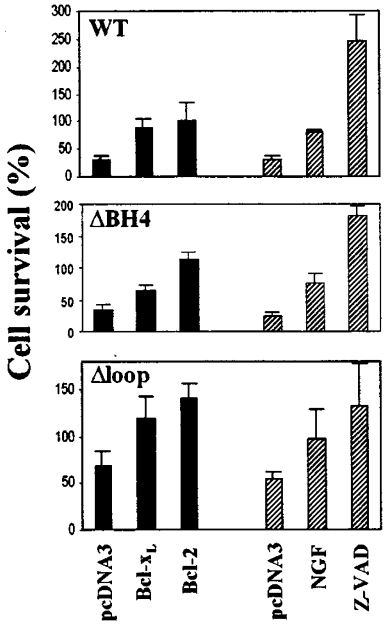

Figure 5 The BH4 and the loop domains of Bcl-x_S are not required for the survival effect of Bcl-x_L, Bcl-2, NGF, or Z-VAD-FMK. PC12 cells were co-transfected for 24 h with SEAP vector together with Bcl-x_S WT (WT) or Bcl-x_S ΔBH4 (ΔBH4) or Bcl-x_S Δloop (Δloop) and pcDNA3 or Bcl-x_L or Bcl-2. In a second set of experiments, PC12 cells were co-transfected for 24 h with SEAP vector together with Bcl-x_S WT and pcDNA3 in the presence or absence of 50 ng/ml NGF or 100 μM Z-VAD-FMK. Cell survival is defined as SEAP activity in each transfection as a percentage of SEAP activity in the corresponding control, i.e., in cells transfected with pcDNA3 or Bcl-x_L or Bcl-2, or with pcDNA3 in the presence or absence of NGF or Z-VAD-FMK. The data shown are mean values ± S.D. (bars) (n=3)

results thus suggest that the BH4 and the loop domains of Bcl-x_S are not required for the survival effect of Bcl-x_L, Bcl-2, NGF, or Z-VAD-FMK in PC12 cells.

Bcl-x_S can form homodimers with itself or heterodimers with Bcl-x_L or Bcl-2

The demonstration that some Bcl-2 family members are able to interact selectively with themselves or with each other suggests that protein interactions between proteins of this family may be an important mechanism for regulating the apoptotic threshold of a cell.[10] It was therefore of interest to determine whether Bcl-x_S-induced cell death and its prevention by the anti-apoptotic molecules involve generation of homodimers by Bcl-x_S with itself or of heterodimers with Bcl-x_L or Bcl-2. PC12 cells were co-transfected with FLAG-tagged Bcl-x_S ΔTM (which, as shown in Figure 2, exhibits diffuse cellular localization) and Bcl-x_S or Bcl-2 or Bcl-x_L expression vectors (which do not contain a FLAG-tagged epitope). The subcellular localization of Bcl-x_S ΔTM was determined 24 h after transfection by staining of the cells with anti-FLAG antibody and immunofluorescence analysis by confocal microscopy (Figure 6). From a set of about 20 fields, about 100 cells that were stained with the anti-FLAG antibody were analyzed in each transfection. In cultures transfected with Bcl-x_S ΔTM alone, all cells that were stained with the anti-FLAG antibody exhibited the characteristic diffuse staining pattern, whereas in cultures transfected with Bcl-x_S ΔTM and Bcl-x_S, Bcl-x_L, or Bcl-2, the staining pattern of most of the cells changed and the diffuse staining pattern was exhibited by only 25, 4, or 19% of the cells, respectively. The rest of the cells showed a more defined staining pattern, which appeared to be largely confined to the mitochondria (Figure 6).

The finding that co-expression of Bcl-x_S ΔTM with Bcl-x_S, Bcl-2, or Bcl-x_L altered the subcellular distribution of Bcl-x_S ΔTM suggests that this change occurs via the interaction of Bcl-x_S ΔTM with Bcl-x_S, Bcl-2, or Bcl-x_L, and this in turn suggests that Bcl-x_S is capable of forming homodimers with itself or heterodimers with Bcl-x_L and Bcl-2. It should be noted that as expected, expression of Bcl-x_S ΔTM by itself did not induce apoptosis in the transfected cells, as indicated by their nuclear morphology. However, cells that were co-transfected with Bcl-x_S ΔTM and Bcl-x_S were mostly apoptotic (as indicated by nuclear condensation and fragmentation), probably because of expression of Bcl-x_S.

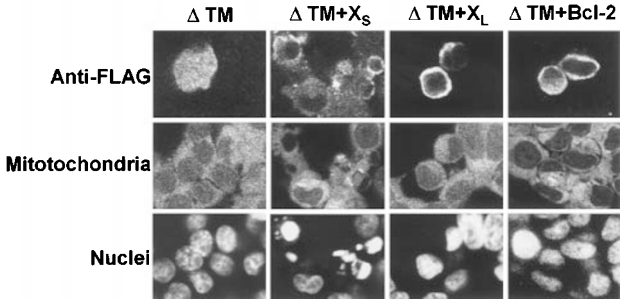

Figure 6 Co-expression of Bcl-x_S ΔTM with Bcl-x_S, Bcl-x_L, or Bcl-2 changes the subcellular distribution of Bcl-x_S ΔTM. PC12 cells were transiently co-transfected with FLAG-tagged Bcl-x_S ΔTM (ΔTM) alone or with Bcl-x_S (X_S) or Bcl-x_L (X_L) or Bcl-2 expression vectors. After 24 h the cells were treated with 250 nM MitoTracker Red and stained with anti-FLAG antibody and Hoechst 33258 (1 μg/ml) (to visualize the nuclei), as described in Figure 3 and Materials and Methods. The figure shows representative images taken by confocal immunofluorescence microscopy ($\times$4800 magnification)

For a more direct test of the ability of Bcl-x_S to form homodimers with itself or heterodimers with Bcl-x_L and Bcl-2, PC12 cells were transfected with FLAG-tagged Bcl-x_S and GFP-Bcl-x_S, Bcl-x_L or Bcl-2. The ability of Bcl-x_S to form homodimers or heterodimers was then examined by the co-immunoprecipitation assay. The generation of Bcl-x_S homodimers was examined in this experiment using GFP-Bcl-x_S in preference to Bcl-x_S, because the difference in molecular weight between Bcl-x_S and FLAG-tagged Bcl-x_S is too small for convenient resolution of these Bcl-x_S forms on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Furthermore, GFP-Bcl-x_S exhibits similar subcellular distribution and apoptotic properties in PC12 cells to those exhibited by Bcl-x_S (data not shown). Transfected cells were harvested 24 h after transfection, and FLAG-tagged Bcl-x_S was immunoprecipitated with the anti-FLAG antibody. Whole cell extract and the immunoprecipitated proteins were subjected to Western blot analysis. As shown in Figure 7A, anti-Bcl-x antibody detected two major bands in the blot, one (25 kDa) corresponding to FLAG-tagged Bcl-x_S and the other (50 kDa) corresponding to GFP-Bcl-x_S. Identification of the 50 kDa band as GFP-Bcl-x_S is further supported by the interaction of this band with anti-GFP antibody. Co-transfection of PC12 cells with FLAG-tagged Bcl-x_S and Bcl-x_S, followed by immunoprecipitation with anti-FLAG antibody and Western blotting with anti-Bcl-x antibody, revealed co-immunoprecipitation of FLAG-tagged Bcl-x_S with Bcl-x_S (data not shown). Taken together, these results strongly suggest that Bcl-x_S is capable of forming homodimers with itself. To examine whether it can also form heterodimers with Bcl-x_L and Bcl-2, PC12 cells were co-transfected with FLAG-tagged Bcl-x_S together with Bcl-x_L or Bcl-2. Cell lysates were immunoprecipitated with anti-FLAG antibody (for FLAG-tagged Bcl-x_S- and Bcl-x_L-transfected cells) or with anti-Bcl-x antibody (for FLAG-tagged Bcl-x_S- and Bcl-2-transfected cells), and Western blotted with anti-Bcl-x or anti-Bcl-2 antibody, respectively. As shown in Figure 7B,C, FLAG-tagged Bcl-x_S can interact with Bcl-x_L or Bcl-2, as each of these proteins co-immunoprecipitated with FLAG-tagged Bcl-x_S. These findings thus suggest that Bcl-x_S is capable of forming heterodimers with Bcl-x_L and Bcl-2.

Discussion

Identification of Bcl-x_S domains required for cell killing and localization to the mitochondria

The results presented here suggest that Bcl-x_S-induced apoptosis is mediated by the localization of Bcl-x_S to the mitochondria via a mechanism that requires the transmembrane domain. They further suggest that the presence of Bcl-x_S in the mitochondria is essential for this protein's pro-

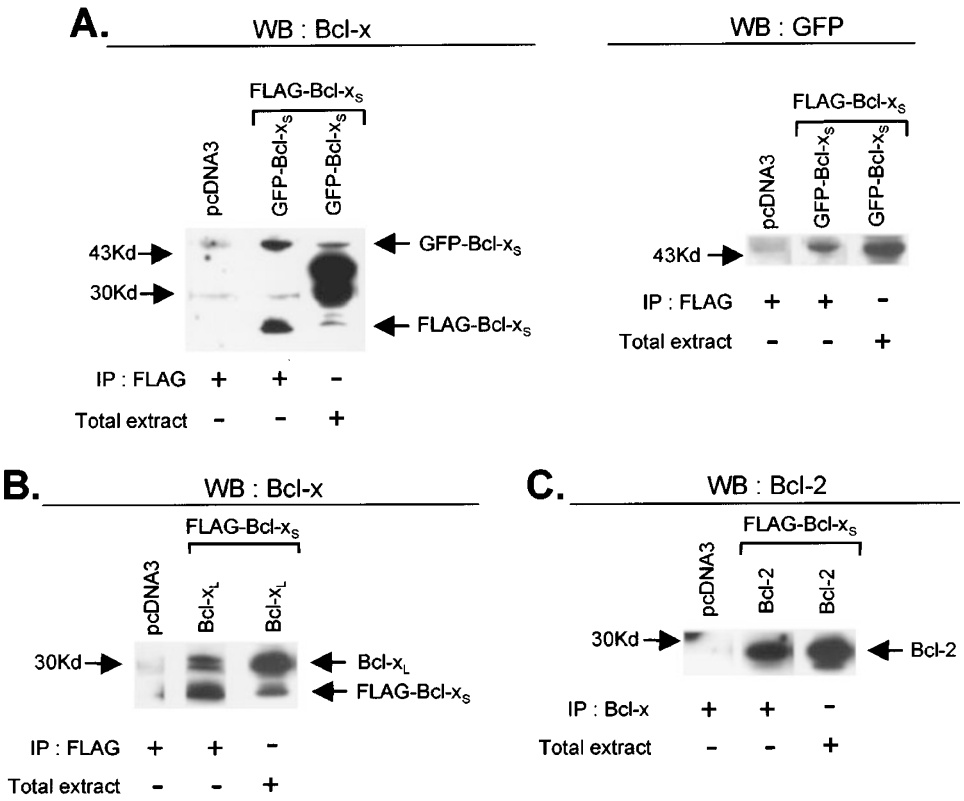

Figure 7 Bcl-x_S can form homodimers and heterodimers. PC12 cells were transiently co-transfected with equal amounts of Flag-tagged Bcl-x_S and GFP-Bcl-x_S, Bcl-x_L, or Bcl-2. Cells were harvested and lysed after 24 h. Whole-cell lysates were immunoprecipitated with anti-FLAG (**A** and **B**) or anti-Bcl-x (**C**) antibodies, and the immunoprecipitates as well as total cell extracts (100 μg protein) were separated by SDS – PAGE and immunoblotted with anti Bcl-x (**A** and **B**), anti-GFP (**A**), or anti-Bcl-2 (**C**) antibodies. WB, Western blot; IP, immunoprecipitation. The data presented are from one representative experiment of three independent experiments

apoptotic function. These conclusions are based on the finding that removal of the transmembrane domain of Bcl-x_S prevents the localization of Bcl-x_S to the mitochondria, as well as the ability of Bcl-x_S to induce cell death. Once Bcl-x_S is present in the mitochondria, its apoptotic effect requires the presence of the BH3 domain and – to a lesser extent – the loop region, as deletion of the two well-conserved amino acids, GD, from the BH3 domain or removal of the loop region did not impair the ability of the Bcl-x_S ΔGD or the Bcl-x_S Δloop mutants to reside in the mitochondria, but blocked (or reduced) their apoptotic effect. The finding that the BH3 domain is needed for the cell death action of Bcl-x_S is supported by previous studies showing that the BH3 domain was needed for reversing the anti-apoptotic function of Bcl-x_L against apoptosis induced by Bax in human embryonic kidney (HEK) 293 cells.[34] The way in which the BH3 domain is involved in cell death induced by the pro-apoptotic members of the Bcl-2 family is unknown. It was suggested that it might participate in the formation of an amphiphatic α-helix that binds with high affinity to the hydrophobic pocket created by the BH1, BH2, and BH3 domains of the anti-apoptotic proteins from the Bcl-2 family, such as Bcl-x_L.[12] In this way, a BH3 domain containing proteins like Bcl-x_S could induce cell death by binding to and antagonizing the effect of the anti-apoptotic family members.

Our findings that the transmembrane and the BH3 domains of Bcl-x_S are important for the apoptotic action of this protein suggest that Bcl-x_S shares at least some elements in its mechanism of action with other anti- and pro-apoptotic members of the Bcl-2 family that were also shown to require the transmembrane domain[21,22,35,36] or the BH3 domain.[23,37–40]

The loop domain of Bcl-x_S may also participate in the death effect. The loop region of anti-apoptotic proteins such as Bcl-x_L has been shown to serve as a site of negative regulation of these proteins.[18] The requirement of the loop domain of Bcl-x_S for this protein's apoptotic effect may thus suggest that in Bcl-x_S, in contrast to Bcl-x_L, this region serves as a site of positive regulation. The mechanism of such positive or negative regulation is still unknown; however, it may involve phosphorylation of sites within the loop, as demonstrated for Bcl-2 and Bcl-x_L.[18] The loop region contains a caspase cleavage site at Asp 61, a site shown to be important for converting anti-apoptotic Bcl-x_L to a pro-apoptotic molecule.[20] Our finding that the D61A mutation did not affect Bcl-x_S-induced cell death demonstrates that such death does not require cleavage of this site. These results suggest that the mechanism whereby Bcl-x_S is activated following an apoptotic trigger differs from that of Bid, another pro-apoptotic member of the Bcl-2 family, in which apoptotic

triggering mediated by death receptors such as CD95 results in caspase-8-dependent cleavage of Bid and translocation of the cleavage product to the mitochondria.[41]

The loop region of Bcl-x_S was previously reported to be dispensable for the pro-apoptotic function of this protein.[34] The discrepancy between that finding and ours may be attributable to differences in the apoptotic systems and cell types employed in these studies. Accordingly, we examined apoptosis induced directly by overexpression of Bcl-x_S in PC12 cells, whereas the earlier study examined the effect of Bcl-x_S on the ability of Bcl-x_L to inhibit Bax-induced cell death in HEK 293 cells.

The BH4 domain is not needed for the apoptotic effect of Bcl-x_S in PC12 cells, as shown by the finding that deletion of this domain did not interfere with the apoptotic effect. This finding is in agreement with previous findings on the effect of Bcl-x_S on the ability of Bcl-x_L to inhibit Bax-induced cell death in HEK 293 cells.[34] The function of the BH4 domain is not known. It was suggested that it might participate in protein–protein interactions between members of the Bcl-2 family as well as between these family members and other regulatory proteins, such as the protein kinase Raf-1,[14] the protein phosphatase calcineurin,[15] Apaf-1,[16] or the cytoplasmic inhibitor protein IκBα, which regulates the function of the transcription factor NF-κB.[42] As our results show that the BH4 domain is not required for Bcl-x_S-induced cell death, they also suggest that the above mentioned regulatory proteins are not involved in promoting Bcl-x_S-induced apoptosis in PC12 cells, at least not via their interactions with the BH4 domain.

Mechanism of action of the anti-apoptotic agents Bcl-2, Bcl-x_L, NGF, and Z-VAD-FMK on Bcl-x_S-induced apoptosis

We have previously demonstrated that Bcl-x_S-induced apoptosis in PC12 cells is inhibited by Bcl-2, Bcl-x_L, the caspase inhibitor Z-VAD-FMK, or NGF.[31] As the results presented here strongly suggest that the presence of Bcl-x_S in the mitochondria is crucial for its apoptotic effect, it might be assumed that these survival factors inhibit Bcl-x_S-induced cell death by preventing the localization of Bcl-x_S to the mitochondria. This seems not to be the case, however, as the present study showed that these survival agents do not prevent the localization of Bcl-x_S to the mitochondria. Previous studies have shown that in Bcl-2 and Bcl-x_L the loop and the BH4 domains may serve as regulatory sites.[18,43] Those findings may thus suggest that in a similar manner the loop and the BH4 domains of Bcl-x_S may be the sites at which the survival agents exert their effects on Bcl-x_S. Our findings suggest, however, that the BH4 or loop domains, as well as post-translational modifications or protein–protein interactions in these domains, are not required for the survival effect of Bcl-2, Bcl-x_L, Z-VAD-FMK, or NGF on Bcl-x_S-induced apoptosis in PC12 cells.

The finding that NGF and Z-VAD-FMK treatments did not prevent Bcl-x_S localization to the mitochondria suggests that the survival effect of NGF acts on the Bcl-x_S apoptotic signaling either at the mitochondria or downstream of the mitochondria, and that Bcl-x_S localization to the mitochondria does not require caspase activity. The dispensability of the caspase cleavage site at D61 in the loop region also suggests that Bcl-x_S localization to the mitochondria does not require caspase activity (at least on this site).

Bcl-x_S can form homodimers with itself and heterodimers with Bcl-x_L or Bcl-2

The present demonstratiaon of changes in the subcellular distribution of Bcl-x_S ΔTM following its co-transfection in conjunction with Bcl-x_S, Bcl-2, or Bcl-x_L, as well as the co-immunoprecipitation of Flag-tagged Bcl-x_S with GFP-Bcl-x_S, Bcl-x_L or Bcl-2, suggests that Bcl-x_S can form homodimers with itself or heterodimers with Bcl-x_L and with Bcl-2. This is the first time that homodimerization of Bcl-x_S has been demonstrated. The assays employed in these experiments are circuitous, and we therefore cannot exclude the possibility that the interaction between Bcl-x_S and the other proteins is indirect, requiring an additional bridging protein(s). The finding of co-immunoprecipitation of Bcl-x_S and Bcl-x_L is in agreement with previous studies carried out in HEK 293 cells.[34] The ability of Bcl-x_S to heterodimerize with Bcl-x_L or Bcl-2 suggests that the pro-apoptotic function of Bcl-x_S may involve binding to these or to other anti-apoptotic members of the Bcl-2 family, thereby inhibiting their survival-promoting activity in the mitochondria. Alternatively, Bcl-x_S might heterodimerize with other pro-apoptotic members of the Bcl-2 family and trigger their apoptotic activity, as shown for Bid, which binds to Bax and thus induces Bax oligodimerization and activation.[44] On the other hand, homodimerization of Bcl-x_S might be sufficient to directly promote cell death activity independently of the anti- or pro-apoptotic members of the Bcl-2 family. This cell death activity may however be prevented by the ability of Bcl-x_L or Bcl-2 to heterodimerize with Bcl-x_S, which in turn will lead to the sequestering and neutralization of Bcl-x_S. Further experiments are needed to determine which of these possibilities mediate the pro-apoptotic effect of Bcl-x_S in PC12 cells.

Materials and Methods

Reagents

NGF was purchased from Chemicon International (Harrow, UK). Benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone (Z-VAD-FMK) was purchased from Enzyme Systems (Dublin, CA, USA), and was resuspended as a 50 mM stock solution in dimethylsulfoxide (Merck, Darmstadt, Germany). Lipofectamine was purchased from Gibco BRL (Life Technologies, Renfrewshire, Scotland). MitoTracker Red CMXRos was purchased from Molecular Probes (Eugene, OR, USA) and was resuspended as a 1 mM stock solution in dimethylsulfoxide. Unless otherwise stated, all other reagents were purchased from Sigma (St. Louis, MO, USA).

Cell culture

PC12 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated horse serum (8%) and fetal calf serum (FCS) (8%).

Plasmids

The Bcl-x_S wild-type (WT) cDNA and various Bcl-x_S mutants were subcloned in frame between the *Eco*RV and *Xho*I sites in a derivative of the pcDNA3 mammalian expression vector that incorporates an N-terminal FLAG (MDYKDDDDK) tag. Bcl-x_S WT, the Bcl-x_S ΔTM mutant (with deletion of the C-terminal residues 150–170), and the Bcl-x_S ΔBH4 mutant (with deletion of residues 4–24) were created by one-step polymerase chain reactions (PCRs) using pBluescript SK(+)Bcl-x_S[13] as a template and 5′-GTCAGATATCTTCTCAGAGCAACCGG-3′ (for Bcl-x_S WT and Bcl-x_S ΔTM), 5′-GTCAGATATCTTCTCAGGGA-TACAGCTGGAGTCAG-3′ (for Bcl-x_S ΔBH4) as the forward primer and either 5′-GTCACTCGAGTCAGCGGTTGAAGCGCTC-3′ (for Bcl-x_S ΔTM) or 5′-GTCACTCGAGTGGTCACTTCCGACT-3′ (for WT Bcl-x_S and Bcl-x_S ΔBH4) as the reverse primer. The Bcl-x_S mutant plamids Bcl-x_S Δloop (with residues 26–83 replaced by three alanines), Bcl-x_S ΔGD (with amino acids 94–95 deleted in the BH3 domain) and Bcl-x_S D61A (with amino acid D61 replaced by A) were prepared by two-step PCR,[45] using pBluescript SK(+)Bcl-x_S as a template and the following mutagenic primers in combination with the Bcl-x_S WT forward and reverse primers described above: Bcl-x_S Δloop, 5′-GCGGCGGCGGCAGCAGTGAAGCAAGC-3′ (forward) and 5′-CGCCGCCGCACTCCAGCTGTATCCTT-3′ (reverse); Bcl-x_S ΔGD, 5′-GAGAGAGGCAGAGTTTGAACTGCGGTAC-3′ (forward) and 5′-GTTCAAACTCTGCCTCTCTCAGCGCTTGCTTC-3′ (reverse); Bcl-x_S D61A, 5′-CACCTGGCGGCTAGCCCGGCC-3′ (forward) and 5′-GGCCGGGCTAGCCGCCAGGTG-3′ (reverse).

GFP-Bcl-x_S expression vector was generated by in-frame subcloning of the *Pst*I-*Bam*HI fragment of Bcl-x_S into these sites in pEGFP-C3 vector (Clontech, Palo Alto, CA, USA). The Bcl-x_S *Pst*I-*Bam*HI fragment was generated by one-step PCR using the pBluescript SK(+)Bcl-x_S as a template, 5′-AAACTGCAGATGTCTCA-GAGCAAC-3′ as the forward primer, and 5′-GCGGATCCTCACTTCC-GACTG-3′ as the reverse primer. The proper construction of all of these plasmids was confirmed by DNA sequencing.

Human Bcl-x_S and Bcl-x_L expression vectors were generated by subcloning the 600 bp and 800 bp *Eco*RI fragments from pBluescript SK(+)bcl-x_S and pBluescript SK(+)bcl-x_L, respectively,[13] into the *Eco*RI site of pcCDNA3. pEGFP-3 (Clontech) encodes a red-shifted variant of wild-type green fluorescence protein (GFP) under the control of the cytomegalovirus (CMV) promoter. pCMV-SEAP was obtained from Dr L Pradier (Rhone-Poulenc Rorer). The vector pcDNA3Bcl-2 was generated in the laboratory of Dr S Korsmeyer (Harvard Medical School, Boston).

Transfection

One day before transfection, PC12 cells were seeded at a density of 2×10^5 cells per well in 24 well plates or 1×10^6 cells per well in 6 well plates. To each 24 well or 6 well plate, 300 μl or 1 ml respectively of DNA-lipofectamine mixture [2 μg DNA and 30 μg lipofectamine in 1 ml of OptiMEM (Gibco BRL)] was added according to the manufacturer's instructions. After incubation of cells for 5 h with the DNA-lipofectamine mixture, DMEM supplemented with 16% serum was added and incubation was continued. For the experiments aimed at examining the effects of NGF and Z-VAD-FMK on Bcl-x_S-induced cell death, the factors were added to the culture medium 5 h after the addition of the DNA-lipofectamine mixture. Viability of the transfected cells in all experiments was monitored, 24 h after transfection, by measuring both the activity of SEAP in the medium of the transfected cells and by examining GFP-positive cells visualized by fluorescence microscopy (data not shown).

The ratios of the different DNA species in each transfection for the cell survival experiments were as follows: 1 : 1 : 1 for Bcl-x_S WT, Bcl-x_S ΔBH4 or Bcl-x_S Δloop/Bcl-2 or Bcl-x_L/SEAP plasmid. In the experiments with Z-VAD-FMK or NGF, the ratio of Bcl-x_S WT, Bcl-x_S ΔBH4 or Bcl-x_S Δloop/pcDNA3/SEAP plasmid was 3 : 1 : 2. Under these conditions different DNA species are taken up by the same cells, as previously demonstrated,[31] by cotransfection of PC12 cells with GFP and blue fluorescent protein (BFP) expression vectors (Clontech). More than 90% of the cells were both GFP- and BFP-positive (data not shown).

The ratios of the different DNA species in each transfection for the immunofluorescence stainings were as follows: 1 : 2 for FLAG-Bcl-x_S WT or FLAG-Bcl-x_S mutants/FLAG-pcDNA3; 1 : 2 for FLAG-Bcl-x_S WT/Bcl-2 or Bcl-x_L; 1 : 2 for FLAG-Bcl-x_S ΔTM/Bcl-2 or Bcl-x_L, and 1 : 1 : 1 for FLAG-Bcl-x_S ΔTM/Bcl-x_S/pcDNA3. In the experiments with Z-VAD-FMK or NGF, the ratio of FLAG-Bcl-x_S WT/FLAG-pcDNA3 was 1 : 2.

The ratios of the different DNA species in each transfection for the immunoprecipitation experiments were as follows: 1 : 1 : 1 for FLAG-Bcl-x_S/GFP-Bcl-x_S/pcDNA3, and 1 : 2 for FLAG-Bcl-x_S/Bcl-2 or Bcl-x_L. In the transfection of FLAG-Bcl-x_S and GFP-Bcl-x_S, Z-VAD-FMK (100 μM) was added to the cultures 5 h after transfection to prevent cell death and thus to increase the amount of Bcl-x_S protein available for immunoprecipitation.

Immunoprecipitation and Western blotting

Immunoprecipitation For each immunoprecipitation, PC12 cells from three wells of 6 well plates were harvested 24 h after transfection and lysed in 0.05 ml cold lysis buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, and 1% CHAPS] supplemented with protease inhibitor cocktail (Calbiochem San Diego, CA, USA). After removal of cellular debris by centrifugation, immunoprecipitation was carried out as follows: lysates were pre-cleared for 30 min at 4°C with 50% anti-mouse or anti-rabbit IgG-agarose beads (Sigma) incubated with 10 μg/ml antibody for 1 h at 4°C, and then for 45 min more with the 50% anti-mouse or anti-rabbit IgG-agarose beads. FLAG-epitope was immunoprecipitated with anti-FLAG M5 mouse monoclonal antibody (Sigma). Bcl-x was immunoprecipitated with anti Bcl-x_S S-18 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blotting The immunoprecipitated proteins or total extracts (100 μg protein) from each treatment were separated by 12.5% SDS–PAGE and electroblotted onto supported nitrocellulose. Uniformity of sample loading was verified by Ponceau staining of the blots. Each blot was blocked for 30 min in 10 mM Tris base, 150 mM NaCl containing 5% fat-free milk, then incubated for 16 h at 4°C with the primary antibody. This first antibody was mouse anti Bcl-2 monoclonal C2 (1 : 1000) (Santa Cruz Biotechnology), rabbit anti Bcl-$x_{L/S}$ S-18 (1 : 1000) (Santa Cruz Biotechnology), or rabbit anti GFP (1 : 1000) (Clontech). Goat anti rabbit (1 : 10 000) or goat anti mouse (1 : 5000) was used as a second antibody. The blots were developed using the Enhanced Chemiluminescence Kit (Amersham, Arlington Heights, IL, USA). For FLAG Western blots, membranes were incubated with M5 mouse monoclonal anti-FLAG antibody (1 : 1000).

Immunofluorescence staining

PC12 cells were grown in 6 well plates, 10^6 cells per well, on coverslips coated with collagen, and transfected as described above. For mitochondrial staining, 24 h after transfection the cells were incubated with 250 nM MitoTracker Red for 15 min at 37°C. The following steps were carried out at room temperature: Cells were washed twice with buffer B [2 mM $CaCl_2$ in Tris-buffered saline (TBS)

×1], fixed with 4% paraformaldehyde for 30 min, washed twice with buffer B, and permeabilized with 0.1% Triton for 10 min. After two more washes with buffer B, cells were incubated with buffer A (2 mM $CaCl_2$, 2% BSA in TBS ×1) with normal goat IgG (200 μg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) to block nonspecific binding. After washing with buffer A, the cells were incubated with buffer A containing anti-FLAG M5 monoclonal antibody (10 μg/ml) (Sigma) for 1 h, then washed three times (10 min each) with buffer A and incubated for 30 min with buffer A containing FITC-labeled goat anti mouse antibodies (2 μg/ml) (Jackson Immuno-Research) preincubated for 1 h with total protein extract powder from PC12 cells to block non-specific binding. After three washes (10 min each) with buffer A, the cells were air dried and mounted with mowiol (Hoechst AG, Germany) containing 29 nM n-propyl gallate (Sigma). For nuclear staining, the cells were subjected to the same procedure except that Hoechst dye 33258 (1 μg/ml) was included in the first wash after the incubation with FITC-labeled goat anti mouse antibodies. Images were collected on a Zeiss LSM 410 confocal microscope equipped with a 25-mW krypton-argon laser (488 nm and 568 nm maximum lines) or a UV laser (364 nm). An oil immersion lens (63 × NA/1.25; Axiovert 135M, Zeiss) was used for imaging.

Assay for SEAP activity

SEAP activity was assayed as described elsewhere.[46] Briefly, culture medium (200 μl per well in 24 well plates) from transfected cells was collected and spun for 2 min at 10 000 × *g*. The supernatant was incubated at 65°C for 10 min and aliquots (25 μl) from each treatment were then incubated with 200 μl of SEAP buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, and 10 mM L-homo-arginine) at 37°C until a yellow color developed. The assay was performed in triplicate for each treatment. The plates were read on a Micro-ELISA reader at a wavelength of 405 nm.

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