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Killing of Sarcoma Cells by Proapoptotic Bcl-X_S: Role of the BH3 Domain and Regulation by Bcl-X_L[1]

Raj. S. Mitra, Mary A. Benedict*, Dalong Qian†, Kimberly E. Foreman‡, Daryoush Ekhterae*, Brian J. Nickoloff‡ and Gabriel Nuñez**

*Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109; †Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109 and ‡Department of Pathology and Skin Disease Research Laboratories, Loyola University Medical Center, Chicago, IL

Abstract

Kaposi's sarcoma (KS) is the most common tumor affecting AIDS patients with over 20% of these patients afflicted by this disease. Previous studies have demonstrated that KS tumor cells predominantly express the prosurvival protein Bcl-X_L compared with Bcl-2. In the current study, we have used an adenoviral vector that expresses Bcl-X_S, a functional inhibitor of Bcl-X_L, to study the significance of Bcl-X_L expression in the KS cell line (SLK) or KS primary cultures. The results demonstrate that 75% to 80% of SLK or KS primary cells were killed by the Bcl-X_S containing adenovirus whereas KS cells infected with control adenovirus showed no significant cell death or growth inhibition. Overexpression of Bcl-X_L, but not Bcl-2, in SLK cells attenuated apoptosis induced by adenovirus Bcl-X_S. Immunoprecipitation experiments revealed that adenoviral Bcl-X_S associated with Bcl-X_L, but not with Bcl-2. Mutational analysis showed that the α2 helical region of Bcl-X_S containing the BH3 motif was critical for killing activity and interaction with Bcl-X_L. These results suggest that Bcl-X_S is a direct killer and Bcl-X_L may act by interacting with and sequestering Bcl-X_S. These studies also suggest that targeting Bcl-X_L may be of therapeutic benefit for the treatment of tumors that are characterized by inappropriate expression of Bcl-X_L. *Neoplasia* (2001) **3,** 437–445.

Keywords: Apoptosis, Bcl-2, Bcl-X, adenovirus, Kaposi's sarcoma.

Introduction

Apoptosis, a common form of programmed cell death, is essential during development and tissue homeostasis and plays a role in the pathogenesis of a variety of diseases [1]. Members of the Bcl-2 family that include prosurvival and proapoptotic proteins have been recognized as key regulators of the apoptotic process [2]. Several members of the family including Bcl-2 and Bcl-X_L have been shown to protect cells from apoptosis induced by a wide array of stimuli, whereas other members of this family such as Bax, Bak, and Bad have been shown to promote cell death [2]. In addition to prosurvival Bcl-X_L, the *bcl-x* gene can encode several protein products including Bcl-X_S as a result of alternative splicing [3–6]. Bcl-X_S is a proapoptotic protein that lacks BH1 and BH2 domains [3], which have been shown to be important in mediating the prosurvival function of prosurvival Bcl-2 family members including Bcl-X_L [7,8]. *bcl-X_S* mRNA levels have been shown to be upregulated before apoptosis associated with mammary tissue involution in mice and ischemia in rat brain [9,10]. Bcl-X_S has been reported to inhibit the protective function of Bcl-2 and Bcl-X_L against apoptosis induced by growth factor deprivation and chemotherapeutic drugs [3,11,12]. Furthermore, expression of Bcl-X_S has been shown to promote apoptosis or nonapoptotic cell death in several experimental systems [13–16].

It has been postulated that overexpression of cell survival proteins may contribute to neoplasia by blocking apoptosis in premalignant or transformed cells. Many cells overexpress Bcl-2 or Bcl-X_L, and this overexpression has been found to correlate with poor prognosis and resistance to treatment in several types of malignancies [17]. Kaposi's sarcoma (KS), a neoplasm with multifocal vascular lesions, is the most common tumor in AIDS patients, afflicting up to 30% of HIV-1–positive patients [18,19]. It has been recently demonstrated that the majority of primary KS tumors overexpress Bcl-X_L [20] but also can express Bcl-2 [21]. As these proteins are known to inhibit cell death, expression of these antiapoptosis Bcl-2 family members in KS cells may contribute to the prolonged survival of the tumor cells *in vivo*.

We previously constructed an adenoviral vector that encodes for the Bcl-X_S protein, and showed that primary carcinoma cells as well as cell lines derived from multiple epithelial tumors were killed following infection with this adenovirus in the absence of an exogenous apoptotic signal [14]. However, the effect of Bcl-X_S expression in sarcoma cells is unknown. Furthermore, the mechanism by which Bcl-

Abbreviations: BH3, Bcl-2 homology domain 3; KS, Kaposi's sarcoma

Address all correspondence to: Gabriel Nuñez, MD, Department of Pathology and Comprehensive Cancer Center, University of Michigan Medical School, 4219 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109. E-mail: bclx@umich.edu

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X_S promotes cell death remains poorly understood. In this report, we demonstrate that Bcl-X_S expression induces apoptosis of KS cells. Significantly, Bcl-X_L but not Bcl-2 attenuated the proapoptotic activity of the *bcl-X_S* adenovirus. Consistent with this finding, Bcl-X_L but not Bcl-2, associated with Bcl-X_S in extracts of KS cells. We also provide evidence that the BH3 motif of Bcl-X_S is critical for Bcl-X_L binding and killing activity, suggesting that Bcl-X_S acts at least in part by antagonizing the prosurvival function of Bcl-X_L.

Materials and Methods

Cells and Cell Culture

KS cell line (SLK) established from a tumor biopsy from oral mucosa of an iatrogenically immunosuppressed HIV-negative man [22] was obtained from Dr. J. Levy (University of California, San Francisco). The cells were maintained in culture using RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. For passage, the cells were treated with 0.05% trypsin–0.53 mM EDTA, washed, and replated. Three different human KS cell cultures were isolated from KS tumors of HIV-positive patients and designated as KS21B, KS23B, and KSL100S as described [23,24]. The other primary KS cell line KSPG38 [25] was obtained from Dr. P. Gill (University of Southern California, Los Angeles). Cells were plated on tissue culture dishes (Corning, Corning, NY) coated with attachment factor (Cell Systems, Kirkland, WA) and were maintained in RPMI 1640 and 20% heat-inactivated fetal bovine serum supplemented with 10% nutridoma HU (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 50 μg/ml endothelial cell growth supplement (ICN Biochemicals, Aurora, OH), and 16 U/ml bovine heparin.

Infection of Cells with Recombinant Adenoviruses

The *bcl-X_S* [14] and control E1-deleted adenovirus [26] were prepared as previously described [14] and the number of adenovirus particles in viral stocks was determined by spectrophotometry. SLK cells were infected with a stock of the beta-galactosidase adenovirus [14] of known titer, and then stained with 5-bromo-4-chloro-3-indolyl β-D-galacto-

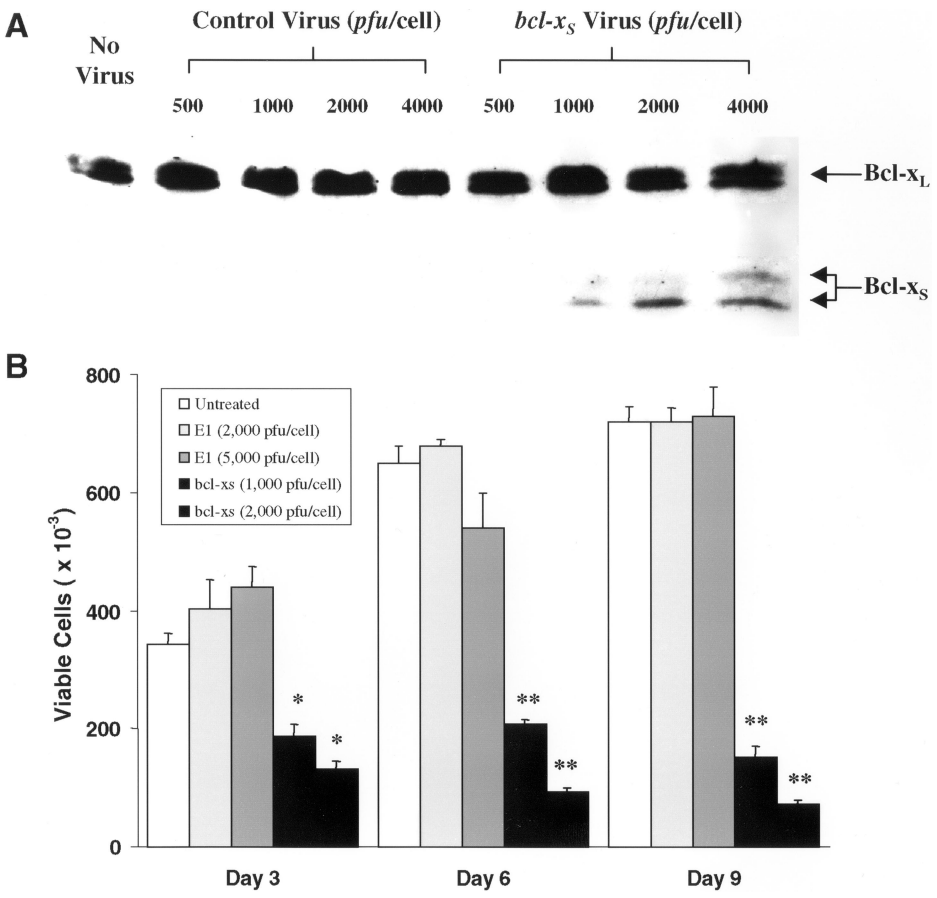

Figure 1. *Adenoviral Bcl-X_S kills SLK cells. (A) Cells were infected either with control or bcl-X_S adenovirus for 72 hours and the expression of Bcl-X_S protein was analyzed by immunoblotting. Note that the cells infected with the virus expressed Bcl-X_S protein whereas the level of endogenous Bcl-X_L protein remained unaltered. (B) SLK cells were infected with adenovirus and at day 3, 6, or 9, the number of viable cells (adhering and nonadhering cells) were determined by trypan blue exclusion. Results are shown as the mean ± SEM of triplicate cultures. */**Indicates P values less than .05/.01, respectively compared with the results of control virus infected cells.*

side to determine the number of viral particles per cell needed to infect approximately 85% to 95% of the cells. For adenoviral infection, SLK cells or KS primary cultures were exposed to the adenovirus vectors for 4 hours in serum-free medium in a small volume sufficient to cover the monolayer culture. Following incubation, 2% serum containing medium was added and incubated overnight. Next day, the medium was removed and replaced with medium containing 10% serum. Each infection was performed at least in triplicate. At the end of desired length of incubation, cell viability of virally infected cell was determined by trypan blue exclusion and cell growth was assessed by hemocytometry. Statistical significance was calculated by Student's paired *t* test. *P* values less than .05 are considered to be statistically significant.

Plasmids

pcDNA3 plasmids containing Flag or HA-tagged *bcl-X_L* or *bcl-2* have been described [27]. Plasmids to express wild-type and mutant Bcl-X_S were generated by a polymerase chain reaction method using appropriate oligonucleotide primers complementary to the human *bcl-X_S* sequence and ligated to pcDNA-HA [27]. The authenticity of the constructs was confirmed by DNA sequencing.

Transfection, Immunoprecipitation, and Immunoblotting

SLK cells were transfected by the calcium phosphate method with 10 μg of the pcDNA3-neo plasmid containing either Flag-tagged *bcl-X_L* or *bcl-2*. Individual cell clones were selected for growth in the presence of G418 (1.5 mg/ml). For immunoprecipitation, cells were lysed in Nonidet P-40 isotonic lysis buffer and lysates were incubated with 1 μg/ml anti-Flag antibody or normal rabbit IgG overnight at 4°C with 5% (vol/vol) of protein A-sepharose 4B (Zymed Laboratories, South San Francisco, CA). Immune complexes were centrifuged, washed four times with excess cold Nonidet P-40 isotonic lysis buffer, separated on a 12% SDS–polyacrylamide gel and immunoblotted with relevant antibodies as described [27]. Rabbit polyclonal anti–Bcl-X (gift from Dr. C. Thompson, University of Pennsylvania) was used for the detection Bcl-X_L and Bcl-X_S protein, whereas Bcl-2 protein was detected by anti–human Bcl-2 monoclonal antibody, 6C8 (Pharmingen, San Diego, CA). Immunoprecipitations to assess the binding of Bcl-X_S mutants to Bcl-X_L were performed in 293 sarcoma cell lysates prepared after transient transfection as described above.

Apoptosis Assay

SLK cells were harvested at day 6 after infection with *bcl-X_S* adenovirus by combining floating cells in the media with adherent cells that were detached with trypsin–EDTA in PBS. Cells were suspended in a hypotonic buffer containing propidium iodide and the percent of cells at sub-Go phase was determined by flow cytometry [28]. For transient assays, wild-type or mutant *bcl-X_S* constructs were cotransfected with a pCMV-βgalactosidase reporter plasmid into 293 kidney sarcoma cells as reported [27]. Apoptosis was determined 18 hours posttransfection in triplicate cultures as reported [27].

Results

Bcl-X_S is Expressed After Infection of SLK Tumor Cells with bcl-X_S Adenovirus

To verify that Bcl-X_S protein is expressed after treatment of the SLK cell line with the *bcl-X_S* adenovirus, extracts from cells infected with various concentrations of control and *bcl-X_S* adenovirus were immunoblotted with an antibody that recognizes both Bcl-X_S and Bcl-X_L. Forty-eight hours after infection, a dose-dependent increase in Bcl-X_S expression was observed (Figure 1*A*). The Bcl-X_S protein was detected as a doublet, which is consistent with a previous report [12]. Bcl-X_S was undetectable in extracts from uninfected cells or cells infected with equivalent titers of control adenovirus (Figure 1*A*). After treatment of SLK cells with control or *bcl-*

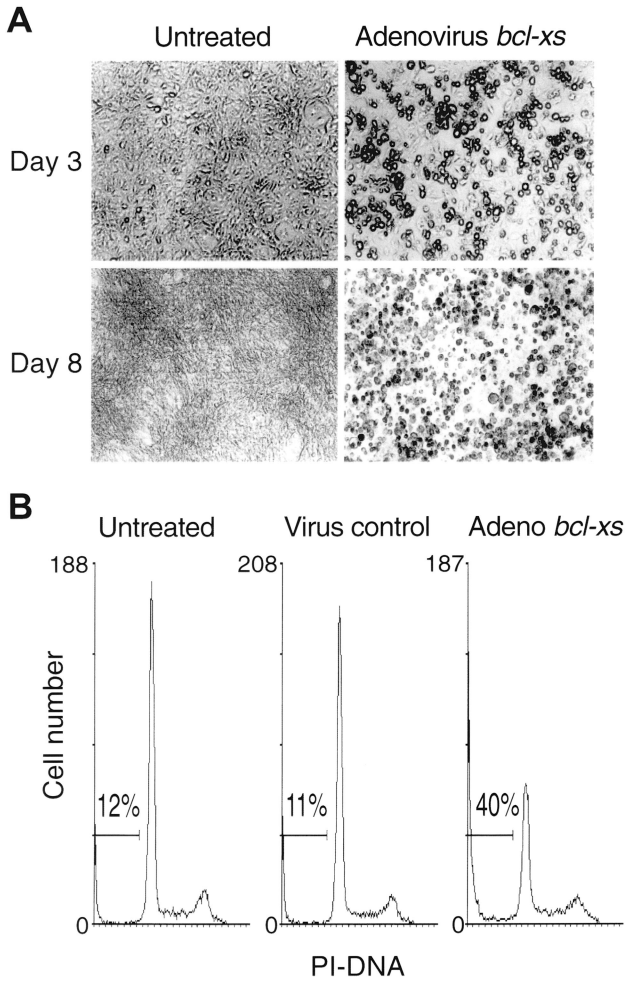

Figure 2. *Viability and morphologic changes of SLK cells following treatment with control adenovirus or bcl-X_S adenovirus (A). Photomicrographs (400×) of a representative field of SLK cells were taken on days 3 and 8 after virus infection. Morphology of cells infected with control adenovirus (not shown) were not different from untreated control. (B) Cell cycle profile was determined in untreated cells, cell infected with control adenovirus, or with bcl-X_S adenovirus using 2000 pfu/cell at 72 hours post infection.*

X_S adenovirus, the levels of endogenous Bcl-X_L were unaffected when compared with those of uninfected SLK cells (Figure 1*A*).

The bcl-X_S Adenovirus Inhibits Cell Growth and Induces Cell Death in SLK Tumor Cells

SLK cells infected with control adenovirus at 2000 or 5000 pfu/cell showed no cytostatic effects on growth as compared with untreated cells when assessed at days 3, 6, and 9 after infection (Figure 1*B*). In contrast, exposure of SLK cells to the *bcl-X_S* adenovirus at 1000 or 2000 pfu/cell resulted in progressive loss of cell growth and reduced cell viability (Figure 1*B*). By day 3 after infection, a significant percentage of the SLK tumor cells infected with the *bcl-X_S* adenovirus displayed rounded-up morphology; by day 8, the great majority of the cells become fragmented and floated in the dish, whereas cells treated with control adenovirus (data not shown) or cells left untreated remained attached to the dish and were viable (Figure 2*A*). Apoptotic cells undergo DNA fragmentation, an event that can be assessed by staining of nuclear DNA [31]. To determine if induction of cell death by the *bcl-X_S* adenovirus was associated with loss of DNA integrity, we performed flow cytometric analysis of nuclei stained with propidium iodide. In a representative experiment, we found that about 40% of the cells infected with the Bcl-X_S adenovirus for 72 hours exhibited a sub-Go DNA profile, which is characteristic of apoptotic cells, as opposed to about 10% apoptotic cells in untreated cultures or SLK cells infected with control virus (Figure 2*B*). These results suggest that the *bcl-X_S* adenovirus induces apoptosis in KS cells.

Bcl-X_S Containing Adenovirus Kills Primary KS Cells

As SLK cells could be killed efficiently by adenovirus *bcl-Xs*, we extended our studies to primary cultures of KS tumor cells to validate our observations made with the KS cell line. Infection of four independent primary cultures of KS tumor cells with the *bcl-X_S* adenovirus resulted in marked cytotoxicity of tumor cells when compared to uninfected cells or cells treated with control adenovirus (Figure 3). To determine the expression levels of Bcl-2 and Bcl-X_L in these KS tumor cells, extracts were prepared and subjected to immunoblotting with anti–Bcl-2 and anti–Bcl-X antibodies. The analysis showed that all the KS tumor cell cultures tested (KS21B, KS23B, KS PG38, and KSL 100S) expressed Bcl-X_L at varying levels, whereas Bcl-2 was detectable only in KS 23B cells (data not shown).

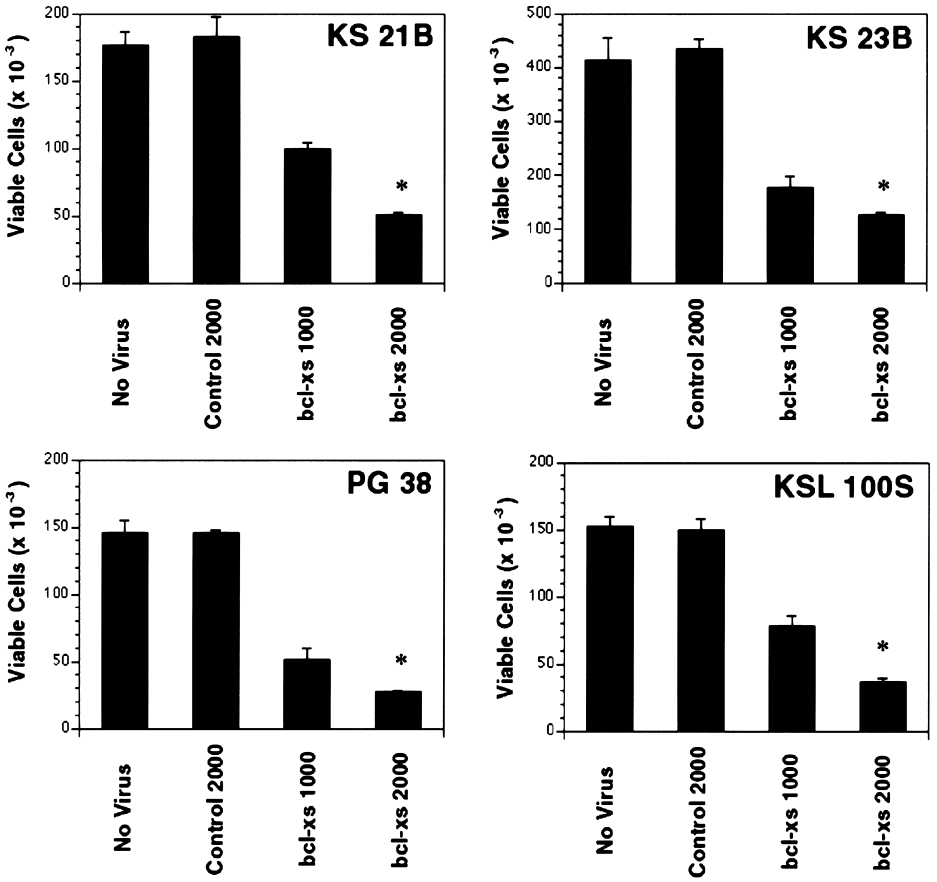

Figure 3. *Cell viability of primary cultures of KS tumor cells infected with bcl-X_S adenovirus and expression of Bcl-X_L and Bcl-2 proteins in primary cultures of KS tumors. KS cells (KS21B, KS23B, PG38, and KSL100S) were infected with either control adenovirus (2000 pfu/cell) or bcl-X_S (pfu/cell indicated in the figure) as described in the Materials and Methods section. At day 8, cell viability of untreated or virally infected cells were determined by trypan blue exclusion and cell numbers were assessed by hemocytometry. *Indicates P values less than .01 compared with cells infected with control virus.*

Bcl-X_L but Not Bcl-2 Protects SLK Tumor Cells from Cell Death Mediated by the bcl-X_S Adenovirus

Primary cultures of KS cells can express Bcl-X_L (Ref. [20] and in this study) and Bcl-2 [21]. To determine if Bcl-X_L or Bcl-2 can regulate killing mediated by the *bcl-X_S* adenovirus, we transfected the SLK cell line with expression plasmids producing Flag-tagged Bcl-X_L, Flag-tagged Bcl-2 or control plasmid and identified two stable clones that overexpress Bcl-X_L or Bcl-2. Flow cytometric analysis using anti-Flag antibody showed that the clones expressed Flag-tagged Bcl-X_L or Bcl-2 in virtually all cells (Figure 4*A*). Western blot analysis confirmed that the clones expressed Flag-Bcl-X_L or Flag-Bcl-2, although the levels of Bcl-2 were higher than those of Bcl-X_L (Figure 4*B*). To determine if exogenous Bcl-X_L and Bcl-2 provide increased survival, SLK cells transfected with plasmids producing these prosurvival proteins or plasmid control were exposed to the chemotherapeutic drug etoposide and cell viability was assessed after drug exposure. Approximately 70% to 75% of the SLK cells lost their viability after incubation with etoposide for 2 days (Figure 4*C*). By contrast, SLK cells overexpressing Bcl-X_L or Bcl-2 showed increased survival after treatment with the drug (Figure 4*C*). To determine whether Bcl-X_L or Bcl-2 could modulate cell killing induced by the *bcl-X_S* adenovirus, SLK tumor cells stably transfected with these prosurvival genes or control plasmid were infected with the *bcl-X_S* adenovirus and cell viability was assessed at different times after adenovirus infection. As seen in Figure 5, expression of Bcl-X_L promoted the survival of SLK cells after infection with the *bcl-X_S* adenovirus whereas Bcl-2 did not.

Bcl-X_L but Not Bcl-2 Interact with Bcl-X_S After Infection of SLK Cells with Adenovirus bcl-X_S

To gain insight into the mechanism by which Bcl-Xs mediates killing of KS cells, we prepared protein extracts from control and adenovirus *bcl-X_S*–infected SLK cells and immunoprecipitated Bcl-X_L and Bcl-2 complexes with anti-Flag antibody. Immunoblotting analysis of control, Bcl-X_L, and Bcl-2 immunoprecipitates with anti–Bcl-X antibody

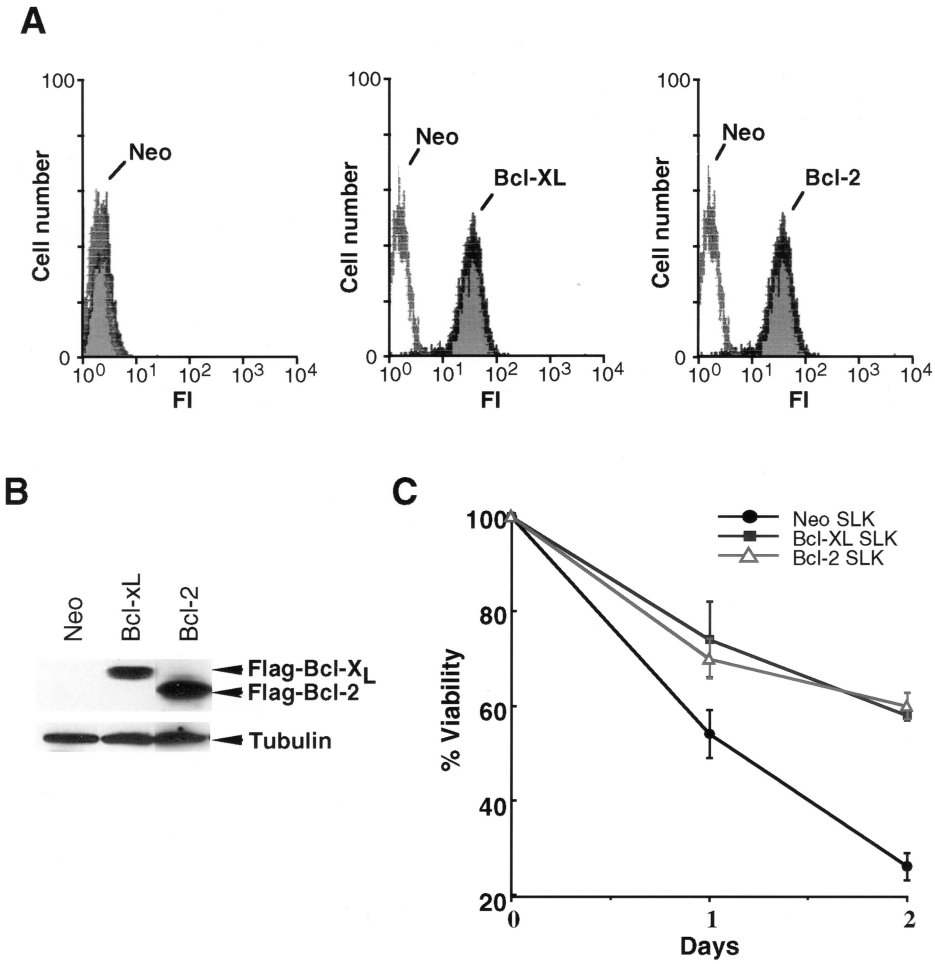

Figure 4. *Expression of functional Bcl-X_L and Bcl-2 in SLK cell clones. (A) Immunoblotting analysis for Flag-Bcl-2 and Flag-Bcl-X_L using anti-Flag monoclonal antibody. Immunoblotting for beta-tubulin was used as a loading control. Note that the SLK-Neo control does not express either Flag-Bcl-X_L or Flag-Bcl-2. (B) Fluorescence histograms of SLK clones. Clones expressing Flag-Bcl-X_L or Flag-Bcl-2 were analyzed for Flag-epitope expression by flow cytometry with anti-Flag monoclonal antibody. Controls are SLK clones expressing Flag-Bcl-X_L or Flag-Bcl-2 stained with isotype-matched control IgG. (C) Cell survival of SLK clones after treatment with etoposide. SLK clones were treated with etoposide (2 μg/ml) and cell viability was determined on days 1 and 2 after continuous exposure to the drug. Results shown are the mean ± SD of triplicate cultures and are representative of at least two separate experiments.*

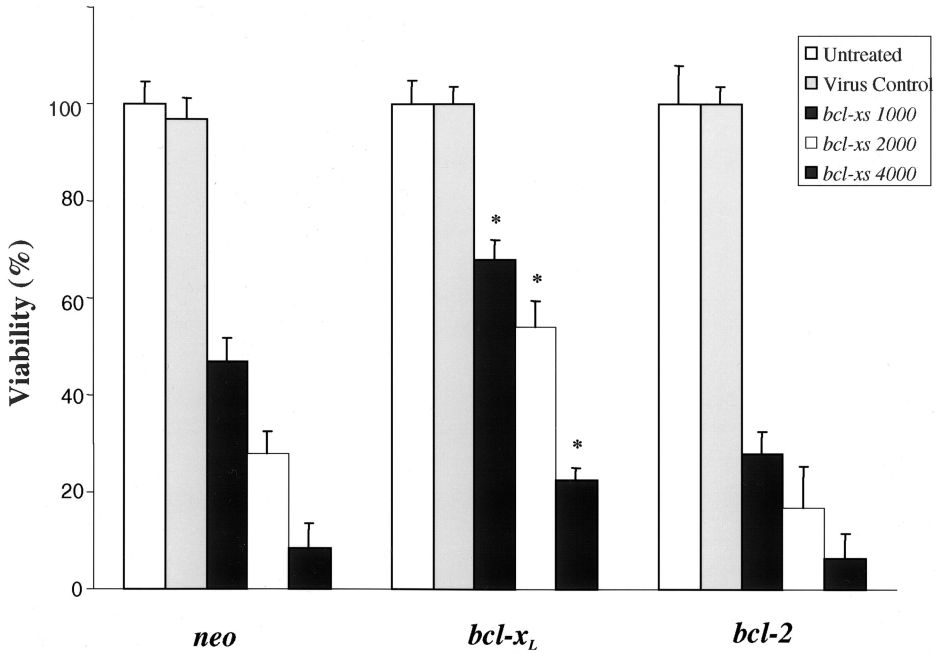

Figure 5. *Viability of SLK cells overexpressing Bcl-X_L or Bcl-2. Cells were infected with control adenovirus or bcl-X_S adenovirus at different pfu/cell (indicated in the figure) and cell viability was determined at day 8. The results are presented as percent of viable cells relative to that of untreated cells that was considered as being 100%. *Indicates P values less than .01 compared with neo-transfected SLK cells.*

revealed that Bcl-X_S associated with Bcl-X_L but not with Bcl-2 (Figure 6). Immunoblotting of total lysates with anti–Bcl-X antibody showed that adenoviral Bcl-X_S was expressed in both Bcl-X_L and Bcl-2 transfectants indicating that the lack of association of Bcl-X_S with Bcl-2 cannot be explained by differential expression of Bcl-X_S in SLK cells (Figure 6).

The BH3 Motif and the C-Terminal Transmembrane Domain of Bcl-X_S are Critical for Killing Activity

We generated a panel of Bcl-X_S deletion mutants based on the known crystal structure of Bcl-X_L [29] to determine the regions that are important for killing activity. These included Bcl-X_S mutants with internal deletion of the BH4 domain (residues 2 to 37), flexible loop region (residues 38 to 62), BH3 domain (residue 86 to 98), most of the α2, α4, and α7 helical region (residues 116 to 132), and the C-terminal transmembrane domain (residues 144 to 170) (Figure 7*A*). Immunoblotting analysis of cell extracts from human 293 kidney sarcoma cells transiently transfected with Bcl-X_S expression constructs revealed that the mutants were expressed in transfected cells (Figure 7*B*). Functional analysis showed that residues 86 to 98 (BH3 domain) and the membrane anchoring domain of Bcl-X_S were required for killing activity (Figure 7*C*). Expression of baculovirus p35, a caspase inhibitor, suppressed apoptosis induced by wild-

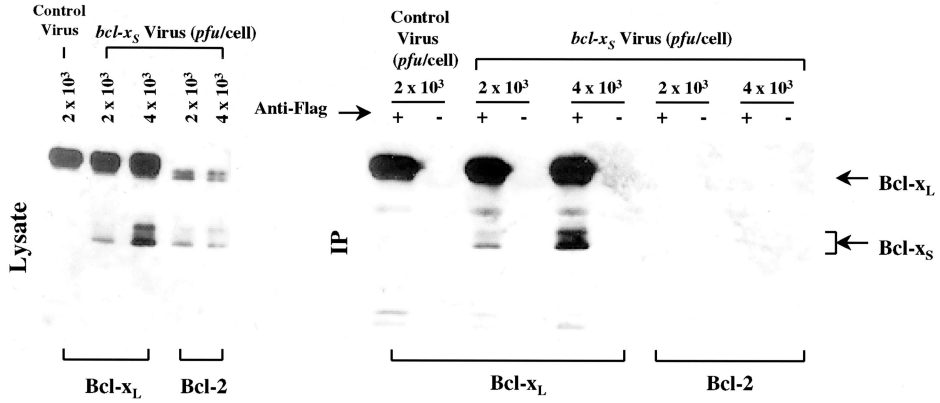

Figure 6. *Differential interaction of Bcl-X_L and Bcl-2 with Bcl-X_S. SLK cells expressing Flag-tagged Bcl-X_L or Bcl-2 were exposed to bcl-X_S adenovirus and for control, SLK cells expressing Flag-tagged Bcl-X_L were exposed to E1-deleted adenovirus (pfu/cell indicated in the figure) for 72 hours. Extracts from infected cells were immunoprecipitated with anti-Flag or control antibody as described in methods and immunoprecipitates were immunoblotted with rabbit polyclonal against Bcl-X.*

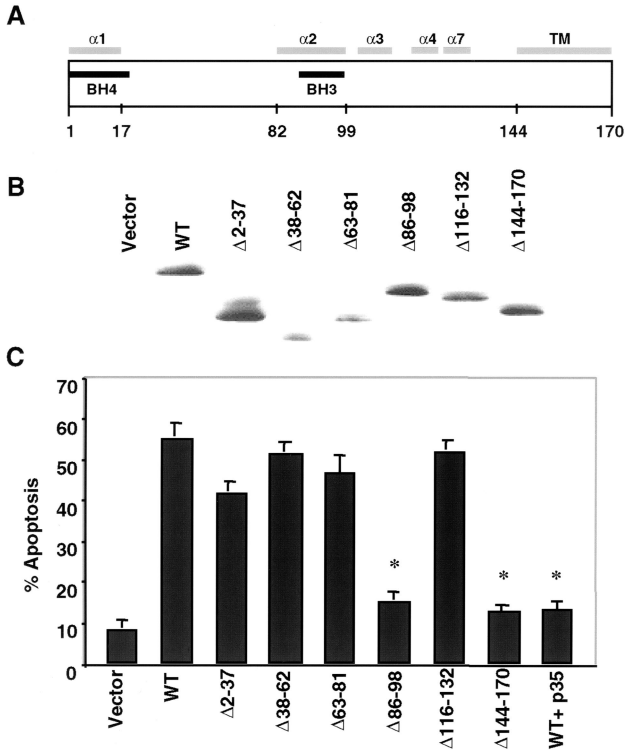

Figure 7. *The BH3 and membrane-anchoring domains of Bcl-X_S are required for proapoptotic activity in 293 kidney sarcoma cells. (A) Schematic diagram of Bcl-X_S structure. Predicted alpha helices and transmembrane region (TM) are shown. Numbers indicate position of amino acids. (B) Immunobloting analysis using extracts of 293 cells transiently transfected with Bcl-X_S plasmids. Expression was detected with anti-HA antibody. (C) Apoptosis in 293 cells transiently transfected with Bcl-X_S plasmids and control plasmid. Assay was performed 28 hours posttransfection. p35 denotes expression of baculovirus p35. Values are means ± SD of triplicate cultures. *Indicates P value less than .01 compared with cells transfected with wild-type plasmid.*

type Bcl-X_S (Figure. 7*C*), suggesting that the proapoptotic activity of Bcl-X_S is mediated through caspases.

The BH3 Domain of Bcl-X_S is Required for Interaction with Bcl-X_L

We next determined the region of Bcl-X_S that is required for Bcl-X_L binding. In these experiments, we coexpressed Bcl-X_S and Bcl-X_L by transient transfection in 293 cells and immunoprecipitated Bcl-X_L with anti-Flag antibody. The analysis in Figure 8 revealed that all mutants except Bcl-X_S (Δ86–98) coimmunoprecipitated with Bcl-X_L, indicating that the BH3 domain of Bcl-X_s is required for the interaction with Bcl-X_L.

Discussion

We report here that KS tumor cells undergo cell death after infection with a replication-deficient adenoviral vector that expresses Bcl-X_S. Adenoviral vector expressing Bcl-X_S caused cell death as early as 3 days after infection of the SLK cell line and several primary cultures of KS cells. Bcl-X_S lacks BH1 and BH2 but contains BH3 and BH4 domains. As it has been shown for BH3-only Bcl-2 family members, such as Bad and Hrk [27,30], the BH3 domain of Bcl-X_S may mediate apoptosis by its ability to heterodimerize, with and inactivate prosurvival Bcl-2 family proteins. Consistent with this hypothesis, our mutational analyses revealed a correlation between Bcl-X_S killing activity and the ability of Bcl-X_s to bind Bcl-X_L. In these experiments, the BH3 domain of Bcl-X_S was required for both Bcl-X_L binding and killing activity. In agreement with our results, it has been shown that only mutant forms of Bcl-X_S that contain the BH3 domain can antagonize the prosurvival function of Bcl-X_L against Bax-mediated apoptosis [12]. The ability of Bcl-X_S to heterodimerize with Bcl-X_L has been controversial [11], although recent experiments support our observation that Bcl-X_S interacts with Bcl-X_L [12]. In contrast, Bcl-X_S did not associate with Bcl-2 in SLK cells infected with the Bcl-X_S adenovirus. Consistent with these findings is our observation that cell death induced by the *bcl-X_S* adenovirus was partially inhibited by Bcl-X_L but not by Bcl-2.

Primary cultures of KS tumor cells or SLK cells, although expressing constitutive levels of Bcl-X_L were susceptible to apoptosis induced by adenovirus Bcl-X_S. Similarly, the *bcl-X_S* adenovirus induced apoptosis in neuroblastoma cells that overexpress Bcl-X_L [31]. It is possible that the constitutive levels of Bcl-X_L may not be sufficiently high to bind all the exogenous Bcl-X_S. However, even after transfection of Bcl-X_L into SLK cells, the *bcl-X_S* adenovirus induced cell death although at a reduced rate compared to controls. Another possibility is that Bcl-X_S is a direct killer that mediates cell death that is in part independent of Bcl-X_L. Although this latter mechanism needs to be further investigated, it may involve binding to mitochondria and disruption of mitochondrial integrity [16], as postulated for other BH3-containing proapoptotic molecules [32,33]. In this model, Bcl-X_L inhibits Bcl-X_S activity by interacting with and sequestering Bcl-X_S. It is most likely that Bcl-X_S acts directly to kill cells, because if it acted through other proapoptotic molecules, such as Bax or Bak, Bcl-2 might be expected to interfere with Bcl-X_S. Our studies have demonstrated that all the primary cultures tested thus far have been effectively killed by the adenovirus *bcl-X_S*. Considering the aggressive nature of AIDS-KS and the lack of currently available effective treatment, inhibition of Bcl-X_L activity may be of benefit for the treatment of this potentially fatal disease. Although the *bcl-X_S* adenovirus may be effective in the treatment of superficial tumors, effective inactivation of Bcl-X_L will require different approaches with improved delivery of therapeutic molecules to tumor cells. Recent studies have identified small molecules that induce apoptosis by binding to the BH3-binding pocket of Bcl-X_L and blocking BH3 domain–mediated heterodimerization between Bcl-2 family members [34,35]. These studies suggest that it may be possible to effectively target the Bcl-X_L survival pathway in diseases such as KS that are characterized by aberrant expression of prosurvival Bcl-2 family members.

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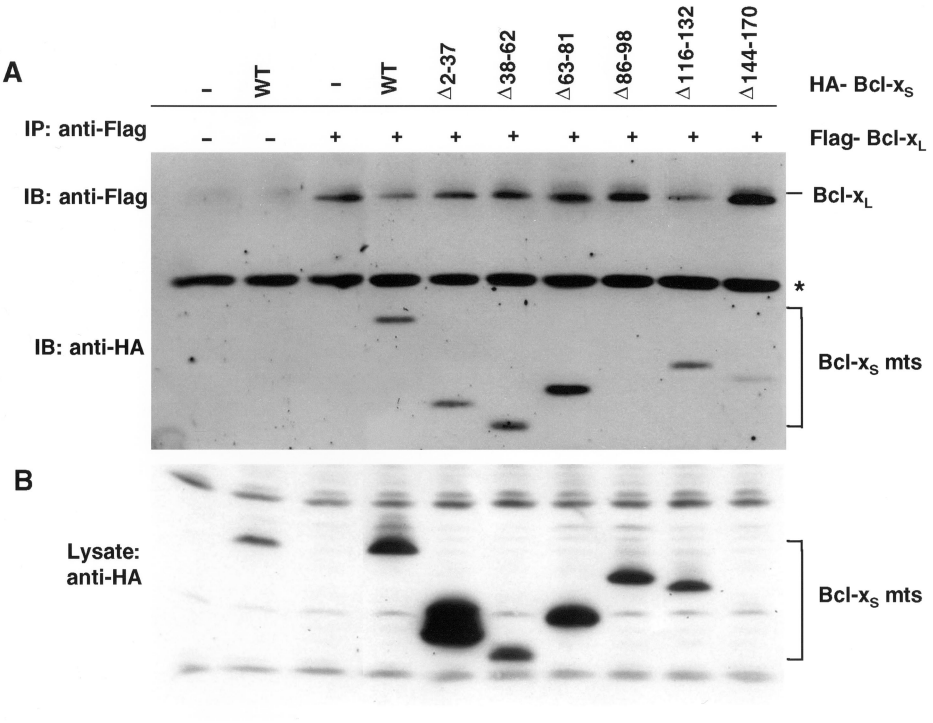

Figure 8. *The BH3 domain is required for binding to Bcl-X_L. (A) Immunoprecipitation of Bcl-X_L with wild-type and mutant Bcl-X_S. 293 cells were cotransfected with plasmids encoding Bcl-X_L and Bcl-X_S as indicated. Bcl-X_L/Bcl-X_S complexes were immunoprecipitated with anti-Flag and immunoblotted with anti-HA and anti-Flag. (C) Immunoblotting of total lysates used for above immunoprecipitations. Note that although deletion mutant Δ144–170 is expressed (see panel A, last lane), it is not visible on this exposure.*

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