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Advances in Brief

Deletion of a Nonconserved Region of Bcl-2 Confers a Novel Gain of Function: Suppression of Apoptosis with Concomitant Cell Proliferation[1]

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Abstract

The Bcl-2 protein coded by the proto-oncogene *bcl-2* is expressed in a variety of embryonic and postnatal tissues and is overproduced in several types of tumors. Bcl-2 expression suppresses apoptosis induced by a multitude of stimuli in diverse cell types without exerting significant effects on cell proliferation, and is believed to contribute to oncogenesis by extending cell survival. In certain B-cell lymphomas, chromosomal translocations result in a gain of function of Bcl-2 by overexpression. Here, we report that a deletion of a nonconserved region of human Bcl-2 (residues 51–85) confers a novel gain of function that not only suppresses apoptosis induced by the tumor suppressor protein p53 and the Myc oncoprotein but also permits continued cell proliferation. Our result raises the possibility that mutations within the *bcl-2* gene may contribute to oncogenesis by both suppressing apoptosis and facilitating cell proliferation.

Introduction

The *bcl-2* gene was discovered as typically involved in the t(14;18) chromosomal translocations observed in human follicular lymphoma (1–3). This chromosomal rearrangement results in deregulated high-level expression of the *bcl-2* gene. In addition, Bcl-2 is also expressed at elevated levels in a variety of other tumors (4–6). The Bcl-2 protein suppresses apoptosis induced by a multitude of stimuli (7, 8). Suppression of apoptosis by Bcl-2, while allowing cell survival, is characterized by proliferation arrest. Although *bcl-2* was discovered as a candidate oncogene, conventional transformation assays indicate that it does not possess dominant oncogenic activity (9). It is therefore believed that unlike other oncogenes, *bcl-2* contributes to oncogenesis primarily by extending cell viability, thereby perturbing the homeostatic mechanisms that control cell number and by providing an environment for other genetic changes (10).

In spite of a lack of detectable autonomous transforming activity, *bcl-2* has been shown to synergize with c-*myc* in the generation of malignant cells (11). Since constitutive expression of c-*myc* induces apoptosis under certain conditions (12–14) that can be suppressed by Bcl-2 (14–16), it appears that the c-*myc*-cooperating oncogenic activity of *bcl-2* may be related to its antiapoptosis activity. In addition, Bcl-2 can also efficiently suppress apoptosis induced by tumor suppressor proteins such as p53 (17–21). This suggests that Bcl-2 may contribute to oncogenesis by suppressing apoptosis induced by oncogenes and tumor suppressor genes.

Although mutations within the Bcl-2 protein that result in a gain of function could play a more direct role (as opposed to deregulated expression) in oncogenesis, thus far no such mutants have been identified in naturally arising tumors or under experimental conditions. Here, we report the construction and characterization of a gain of function mutant of human Bcl-2 that retains the ability to suppress apoptosis induced by the p53 tumor suppressor protein and Myc oncoprotein, while allowing concomitant cell proliferation.

Materials and Methods

Plasmids. Plasmid pRcCMV-Bcl2 was constructed by cloning the human *bcl-2* gene (22) into the *Hin*dIII and *Xba*I sites of the mammalian expression vector pRcCMV (Invitrogen). Mutant Bcl2Δ51–85 was constructed by PCR mutagenesis using a mutagenic oligonucleotide primer 5′-GGA-CCA-CAG-GTG-GCA-CCG-GGC-TGA-GGC-TAG-CGG-AGA-AGA-AGC-CCG-GTG-CGG-GGG-CG-3′ and two other primers complementary to the 5′ and 3′ ends of *bcl-2*. This mutagenesis introduces an *Nhe*I site and substitutes an alanine and a serine residue in the deleted region. The PCR product was cloned into the *Hin*dIII and *Xba*I sites of pRcCMV to generate pRcCMV-Bcl2Δ51–85. pTM1-based plasmids expressing *wt*[3] Bcl-2 and mutant Bcl2Δ51–85 were constructed by cloning the respective genes into the *Nco*I and *Sal*I sites of the vector pTM1 (23).

Cell Lines. The BRK-p53val135-E1A cell line has been described (21) and was maintained at 38.5°C in DMEM supplemented with 10% FCS. BRK-p53val135-E1A cells stably expressing Bcl-2 were generated by transfection of various pRcCMV-based Bcl-2 expression plasmids and selection with G418 (250 μg/ml). Rat1a and Rat1MycER-Hygro cells have been described (14, 24). Cells expressing ER fusion proteins were maintained in DMEM without phenol red and 10% FCS (certified low estrogen content; Life Technologies, Inc.). Rat1MycER-Hygro cells expressing Bcl-2 were selected by transfection with pRcCMV-Bcl2 or pRcCMV-Bcl2Δ51–85 and selection with 400 μg/ml G418. DNA transfections were carried out using the standard calcium phosphate method.

Cell Death Assays. BRK-p53val135-E1A cells were plated at 5×10^5 cells/35-mm dish. After 12 h at 38.5°C, the dishes were shifted to 32.5°C, and at various intervals cells were trypsinized in triplicates, stained with 0.2% trypan blue, and viable cells were counted. Rat1-Hygro cells (2.5×10^5) were plated in 60-mm dishes, incubated for 12 h at 37°C, washed three times in serum-free DMEM, and maintained in fresh media containing 0.1% FCS and 1 μM β-estradiol. Cell viability was determined at various intervals.

Immunoprecipitation. Bcl-2 or Bcl2Δ51–85 proteins were coexpressed with HA epitope-tagged Bax using the vaccinia virus/T7 coupled expression system as described previously (25). BSC40 cells were transfected with pTM1 expression plasmids using LipofectAMINE (Life Technologies, Inc.) and infected with the recombinant vaccinia virus vTF7-3 (23) expressing the T7 RNA polymerase. Sixteen h postinfection, cells were metabolically labeled with 500 μCi [^{35}S]methionine and [^{35}S]cysteine mixture for 2 h and lysed in isotonic buffer (25) containing protease inhibitors (0.04 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 200 μM phenylmethylsulfonyl fluoride). Lysates were precleared with protein A-Sepharose for 1 h, which was removed by centrifugation. The proteins were immunoprecipitated with a rabbit polyclonal antibody specific for human Bcl-2 (prepared against glutathione *S*-transferase/full-length Bcl-2 fusion protein) or with HA monoclonal antibody (12CA5; Boehringer Mannheim). The proteins were analyzed using electrophoresis on 13% SDS-polyacrylamide gels and detected using fluorography.

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[3] The abbreviations used are: *wt*, wild type; ER, estrogen receptor; HA, influenza virus hemagglutin epitope; BRK, baby rat kidney.

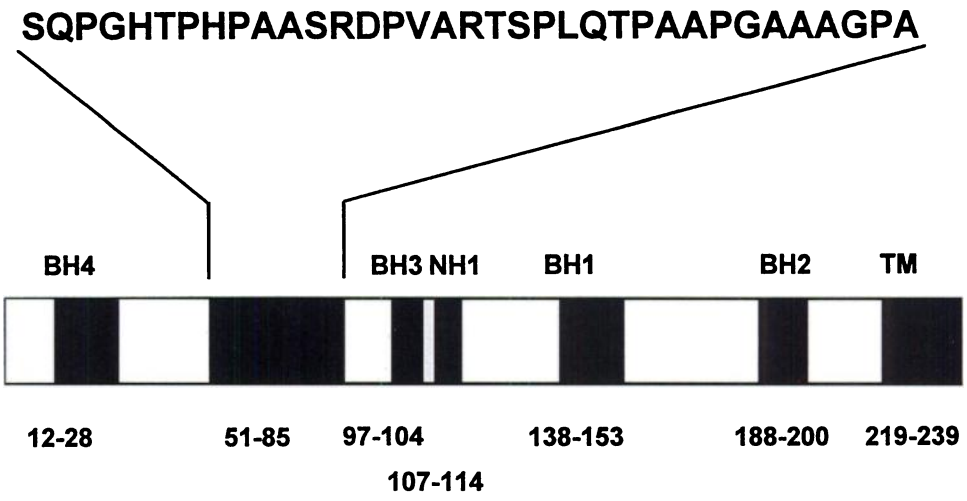

Fig. 1. Domain structure of Bcl-2. The various conserved domains (BH1–4) are indicated. BH1–3 are conserved among both survival-promoting and death-promoting members of the Bcl-2 family of proteins. BH1 and 2 are described in Ref. 33. BH3 is described in Ref. 32. BH4 is conserved among survival-promoting members and corresponds to box A described by Sato *et al.* (39). NH1 is described in Ref. 21. *TM*, transmembrane domain. The sequences deleted in mutant Bcl2Δ51–85 are indicated.

Results and Discussion

Effect on p53-induced Apoptosis. We first chose to examine a nonconserved region located between residues 51 and 85 (Fig. 1) with the rationale that such sequences may regulate the activity of Bcl-2. Deletion of this region of Bcl-2 (Bcl2Δ51–85) did not significantly alter the level of expression of the mutant protein (Figs. 2*A* and 3*A*). We tested the effect of Bcl-2 *wt* and mutant Bcl2Δ51–85 on apoptosis induced by the tumor suppressor protein p53 (26). BRK cells transformed with adenovirus E1A and a *ts* mutant of p53 (p53val135; Ref. 27) express very high levels of mutant p53 at the nonpermissive (38.5°C) temperature and undergo rapid apoptosis after the p53 protein assumes *wt* conformation at 32.5°C (28). This apoptosis can be

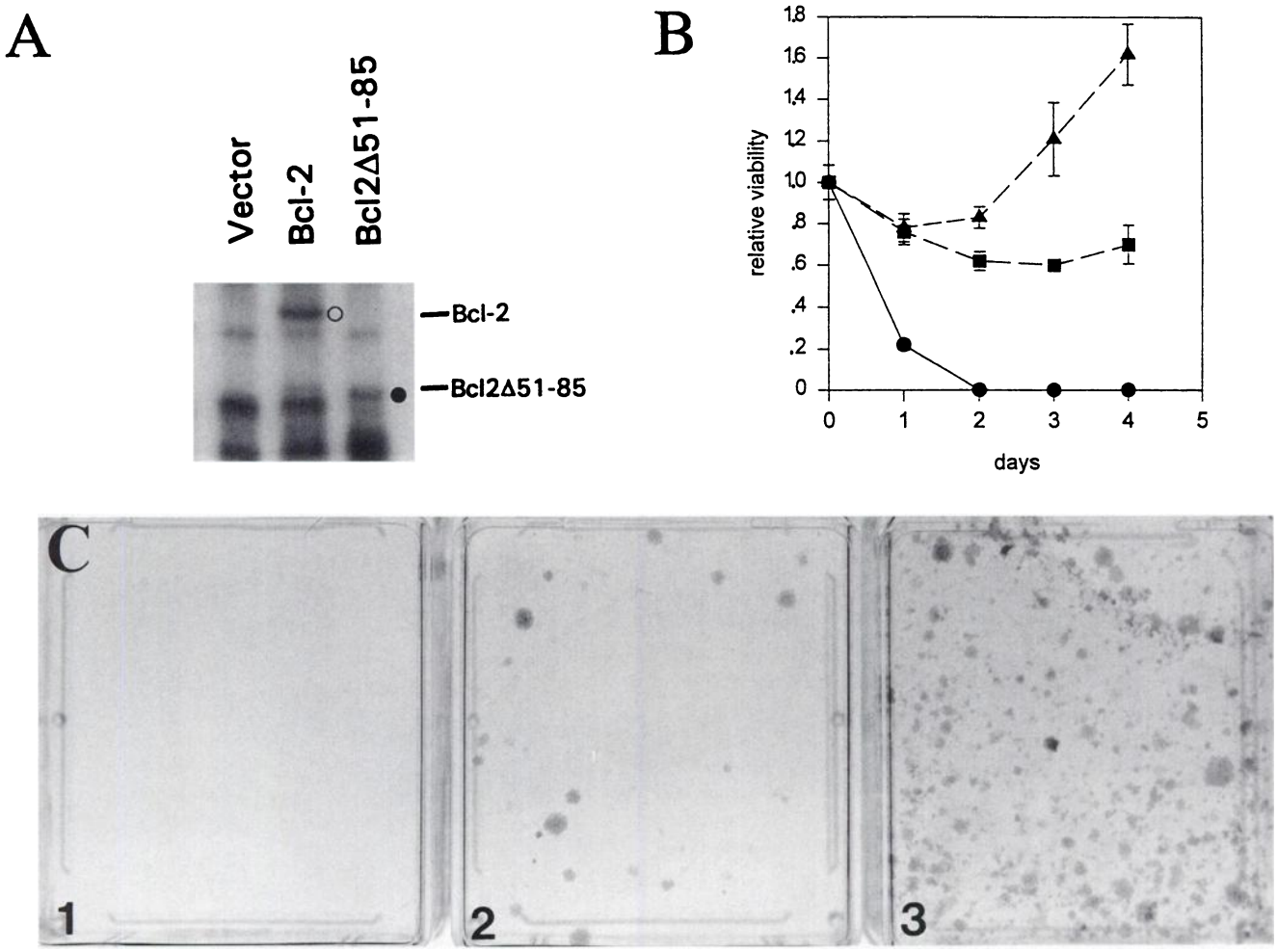

Fig. 2. Suppression of p53-induced apoptosis by Bcl-2. *A*, immunoprecipitation analysis of Bcl-2 and Bcl2Δ51–85 expression in BRK-p53val135-E1A cells. *B*, survival/proliferation of BRK-p53val135-E1A cells at 32.5°C. ●, pRcCMV vector; ■, *wt* Bcl-2; ▲, Bcl2Δ51–85. *C*, long-term proliferation of BRK-p53val135-E1A cells. *1*, pRcCMV vector; *2*, *wt* Bcl-2; *3*, Bcl2Δ51–85.

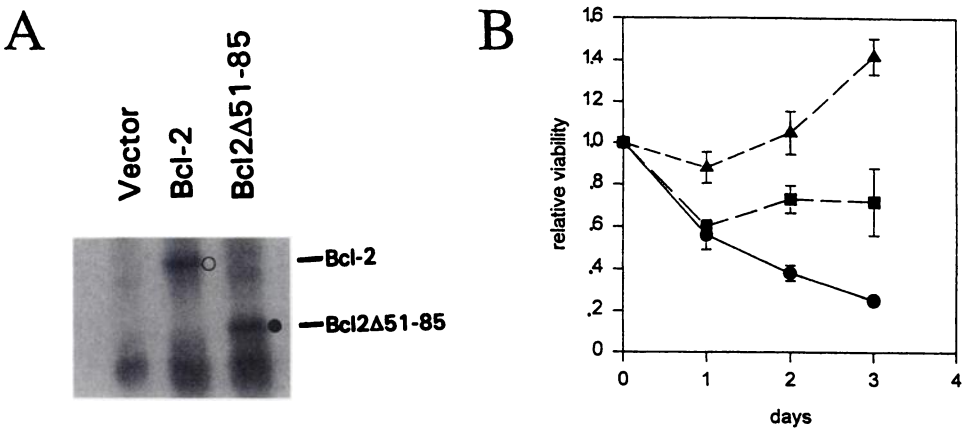

Fig. 3. Suppression of Myc-induced apoptosis by Bcl-2. *A*, immunoprecipitation analysis of Bcl-2 and Bcl2Δ51–85 expression in Rat1MycER-Hygro cells. *B*, survival/proliferation of Rat1MycER-Hygro cells. ●, pRcCMV vector; ■, *wt* Bcl-2; ▲, Bcl2Δ51–85.

efficiently suppressed by Bcl-2 (20). BRK-p53val135-E1A cells were transfected with the pRcCMV vector or pRcCMV-Bcl2 or pRcCMV-Bcl2Δ51–85, and G418-resistant colonies were selected at 38.5°C. Comparable numbers of colonies were pooled and assayed for survival at 32.5°C. As expected, *wt* Bcl-2 efficiently suppressed cell death compared to cells transfected with pRcCMV vector (Fig. 2*B*). Cells expressing Bcl2Δ51–85 did not lose cell viability significantly at 32.5°C. Interestingly, these cells also proliferated efficiently at this temperature in contrast to cells expressing *wt* Bcl-2 (Fig. 2*B*). The observed effect of mutant Bcl2Δ51–85 is probably an underestimate considering that steady-state levels of the mutant protein, estimated using immunoblot analysis, was generally lower than that of *wt* Bcl-2 (data not shown). In addition to mutant Bcl2Δ51–85, we have also examined the effect of several other substitution and deletion mutants in various conserved regions of Bcl-2 (data not shown). All other mutants examined were either totally defective in cell survival activity or exhibited some limited effect. These mutants are not discussed further here.

The effect of mutant Bcl2Δ51–85 on long-term proliferation was also determined. Pooled cell lines transfected with Bcl-2 *wt* or Bcl2Δ51–85 or pRcCMV vector were plated at low cell density, maintained at 32.5°C for 3 weeks, and stained with Giemsa (Fig. 2*C*). Cells transfected with pRcCMV died rapidly without forming any detectable colonies. Cells transfected with *wt* Bcl-2 survived for an extended period, but formed very few proliferating colonies. Consistent with their behavior in short-term cell survival/proliferation assays (Fig. 2*B*), cells transfected with mutant Bcl2Δ51–85 formed numerous proliferating colonies. These results indicate that the mutant Bcl2Δ51–85 facilitates long-term proliferation of cells under conditions that otherwise result in apoptosis.

Effect on Myc-induced Apoptosis. We also tested the effect of Bcl2Δ51–85 on Myc-induced apoptosis. Rat1 cells expressing the c-*myc* gene fused to the human ER (mycER-Hygro) undergo apoptosis after Myc expression is activated by addition of β-estradiol and cells are deprived of serum (13, 14). The mycER-Hygro cells were transfected with pRcCMV vector or pRcCMV-based plasmids expressing *wt* Bcl-2 or mutant Bcl2Δ51–85, and pooled G418-resistant cell lines were established. Immunoprecipitation (Fig. 3*A*) and protein blot (data not shown) analyses revealed that the various Rat1 cell lines expressed comparable levels of *wt* or the mutant Bcl-2 proteins. The effect of Bcl-2 expression on Myc-induced apoptosis was then determined by treating the cells with 1 μM β-estradiol in media containing 0.1% FCS. As expected from previous reports, deregulated Myc expression induced significant cell death. Expression of *wt* Bcl-2 resulted in about 60% cell survival. As in the case of BRK/p53val135-E1A cells, the expression of the Bcl2Δ51–85 mutant not only suppressed cell death but also induced significant proliferation of mycER-Hygro cells in low serum after a lag period of about 1 day (Fig. 3*B*).

Interaction of Cellular Proteins with Bcl2Δ51–85. To determine whether deletion of the amino acid region encompassing residues 51–85 affected interaction of various cellular proteins, we examined the interaction of several cellular proteins that have been previously reported to interact with Bcl-2 either by two-hybrid interaction studies in yeast (29) or using coimmunoprecipitation analyses. In these studies, we observed that there was no major difference in the patterns of interaction of Nip1–3 (25), c-Raf-1 (30), Bak (31), and Bik (32) (data not shown). In contrast, the level of interaction between Bax (33) and Bcl2Δ51–85 appears to be significantly enhanced in comparison to *wt* Bcl-2 in coimmunoprecipitation assays (Fig. 4). This enhanced interaction appears to be significant considering that the total level of Bax was similar in cells expressing either Bcl2Δ51–85 or *wt* Bcl-2.

Thus, we have identified a deletion mutant of Bcl-2 that has a novel activity. The deletion mutant Bcl2Δ51–85 not only suppresses apoptosis induced by the tumor suppressor protein p53 and the Myc onco-protein, but unlike *wt* Bcl-2, facilitates continued cell proliferation. We believe that our results may have important implications for oncogenesis involving Bcl-2. Unlike other oncogenes, the *bcl-2* proto-oncogene promotes cell survival without significant cell proliferation.

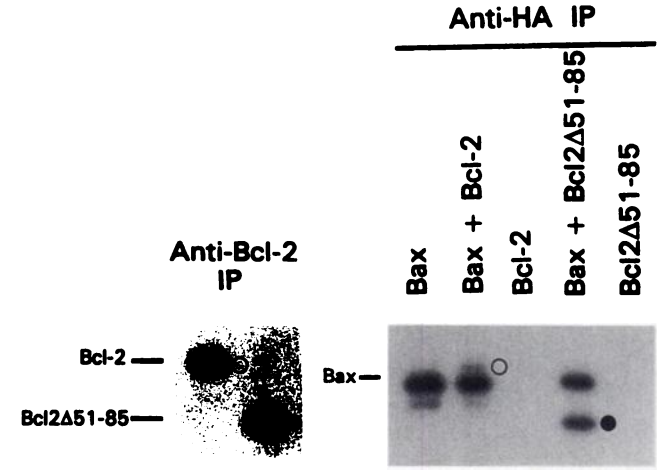

Fig. 4. Interaction of Bax with Bcl-2 and Bcl2Δ51–85. BSC40 cells were transfected with pTM-HA Bax and pTM-Bcl-2 or pTM- Bcl2Δ51–85 and infected with vaccinia virus vTF7–3. ^{35}S-labeled proteins were immunoprecipitated either with HA mouse monoclonal or Bcl-2 rabbit polyclonal antibody and analyzed on 13% SDS-polyacrylamide gels. The identity of coprecipitating Bcl-2 was determined using protein blot analysis of the same sample.

Our results suggest that certain mutations can activate a proliferation-facilitating activity. Although we have not examined the effect of Bcl2Δ51–85 against a wide array of apoptotic stimuli, the observed effect against oncogene/antioncogene-induced apoptosis may potentially prove to be of considerable significance in oncogenic events involving Bcl-2. Activating mutations within the nonconserved region may enhance tumorigenesis by antagonizing the apoptotic activities of p53 and Myc as well as by facilitating continued cell proliferation. Our results also point out a region of Bcl-2 that should be examined closely for activating mutations in various human tumors.

The molecular basis for this gain of function in the Bcl-2 mutant is not clear. Clearly, additional mutagenesis of the nonconserved region is needed to identify the sequence motif(s) involved in regulation of Bcl-2 activity. Our results suggest that the gain of function does not correlate with the ability of Bcl-2 to interact with several proteins. However, the interaction between Bcl-2 and the death-promoting protein Bax appears to be enhanced. It is not clear whether this enhancement is due to higher affinity or increased stability of the Bcl-2/Bax complex. Additional study is required to establish a correlation between Bax interaction and the gain of proliferation activity in cells expressing the Bcl-2 mutant. The region deleted in Bcl2Δ51–85 contains several serine-threonine residues. It has been reported that Bcl-2 activity can be negatively modulated by phosphorylation (34). Deletion of the potential phosphorylation sites could unmask the novel Bcl-2 activity. Alternative explanations to account for the mutant phenotype are also possible. The deleted region is rich in alanine and proline residues. The possibility that these residues play some negative regulatory role in Bcl-2 activity remains to be investigated. Interestingly, the nonconserved region deleted in the mutant is absent in the EBV BHRF1 protein which has antiapoptosis activity like the *wt* Bcl-2 protein (35–37). BHRF1 (*wt*) does not exhibit the proliferation-promoting activity analogous to Bcl2Δ51–85, and mutations elsewhere in BHRF1 activate a proliferation-facilitating activity (38). It therefore appears that the gain of function phenotype that we have discovered in Bcl-2 may be the result of interplay with other functional domains of Bcl-2.

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