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Advances in Brief

bfl-1, a *bcl-2* Homologue, Suppresses p53-induced Apoptosis and Exhibits Potent Cooperative Transforming Activity[1]

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Abstract

The *bcl-2* family of genes code for proteins that contain anti-apoptotic or pro-apoptotic activity. The human *bfl-1* gene contains an open reading frame for a 175-amino acid Bcl-2 family protein. Among the various Bcl-2 family members, the Bfl-1 protein shares the highest homology with the mouse A1 protein. These two proteins share three conserved domains, Bcl homology (BH)1, BH2, and BH3, with other Bcl-2 family proteins. Unlike other Bcl-2 family members, Bfl-1 contains a Gln-rich NH_2-terminal region and lacks an NH (19K homology) domain 1. We demonstrate that the Bfl-1 protein suppresses apoptosis induced by the p53 tumor suppressor protein in a manner similar to other Bcl-2 family members such as Bcl-2, Bcl-x_L and EBV-BHRF1. In addition, the *bfl-1* gene cooperates efficiently with the *E1a* oncogene in transformation of primary rodent epithelial cells. Our results suggest that the human *bfl-1* gene may play an important role in carcinogenesis.

Introduction

During the past few years, a number of cellular and viral proteins that are structurally related to the protein coded by the proto-oncogene *bcl-2* have been identified. Certain members of the Bcl-2 family such as Bcl-x_L (1), EBV-BHRF1 (2–4), MCL-1 (5), and A1 (6) promote cell survival by suppressing apoptosis in a fashion analogous to the founding member of this family, Bcl-2 (reviewed in Refs. 7 and 8). In contrast, other members of the family promote cell death. The death-promoting members of the Bcl-2 family include Bax (9), Bak (10–12), and Bik (13). Similarly, two family members that antagonize the anti-apoptotic activity of Bcl-2 family proteins have been described. Bcl-x_S, a splice variant of Bcl-x, acts against Bcl-2 and Bcl-x_L (1, 14), whereas Bad antagonizes the activity of Bcl-x_L (15).

In addition to these *bcl-2* family members, other related genes whose apoptosis-regulating activities are unknown have also been described. These include the *LMW5-HL* gene (16) of African swine fever virus, Herpes virus saimiri *ORF16* (17), quail *NR-13* (18), and human *bfl-1* (19). Among the various *bcl-2* family genes, the *A1* and *bfl-1* genes may be considered as homologues since the proteins coded by these genes share about 72% amino acid identity. The *A1* gene was identified as an early-response gene in hematopoietic cells treated with granulocyte-macrophage colony-stimulating factor (20). More recently, the *A1* gene, like *bcl-2*, has been shown to extend the survival of myeloid precursor cells after growth factor withdrawal (6). But unlike *bcl-2*, *A1* permitted differentiation of these myeloid cells. These results suggest that A1 may share some functional similarity with Bcl-2 while also possessing some distinct activities. The human *bfl-1* gene was discovered during computer searches of the cDNA databases (19). *bfl-1* is expressed at elevated levels in normal bone marrow and in several clinical samples of stomach cancer (19).

An examination of the amino acid sequence of the Bfl-1 protein reveals that it contains the highly conserved BH1 and BH2 domains (9) and the recently recognized BH3 domain (13, 21, 22), like other Bcl-2 family proteins (Fig. 1). Despite these homologies, Bfl-1 does not contain a well-defined COOH-terminal *trans*-membrane domain, which is characteristic of the Bcl-2 family proteins. Further, Bfl-1 lacks a domain designated as NH1 (23) that is essential for the anti-apoptosis activity of adenovirus E1B-19 kDa protein (24), Bcl-2 (23), and EBV-BHRF1 (25). Bfl-1 appears to have a cryptic BH4 domain that is essential for the anti-apoptosis activity of Bcl-2 (26, 27), Bcl-x_L (14), and EBV-BHRF1 (25). However, the limited BH4 homology observed in Bfl-1 does not extend to A1 (Fig. 1). Interestingly, the NH_2-terminal region of both Bfl-1 and A1 contain several Gln residues. These unique structural features may contribute to functions that are distinct from other Bcl-2 family proteins.

In this communication, we report that Bfl-1 suppresses apoptosis induced by the p53 tumor suppressor protein in a manner similar to Bcl-2, Bcl-x_L, EBV-BHRF1, and adenovirus E1B-19 kDa proteins. In addition, we demonstrate that the *bfl-1* gene has a potent dominant cooperating oncogenic activity in transformation of primary rodent epithelial cells.

Materials and Methods

Plasmids. The plasmid pcDNA3HA-Bfl-1 was constructed by cloning the PCR-generated Bfl-1 open reading frame into the vector pcDNA3HA, which contains the influenza virus HA[4] epitope. The DNA fragment containing the Bfl-1 open reading frame was generated by PCR from a human B-cell cDNA library using two primers, 5′-GGCGGATCCGACAGACTGTGAATTTG-GATATATT-3′ and 5′-CCGCTCGAGTCAACAGTATTGCTTCAGGAG-AGA-3′. Two independent (pcDNA3HA-Bfl-1) clones were sequenced and were found to contain DNA sequences (within the Bfl-1 open reading frame) identical to the sequences reported previously (19). Plasmids pRcCMV-Bcl2 (23), pRcCMV-BHRF1 (4), pRcCMVHA-Bcl-x_L (13), and pRcCMV-19K (24) have been described.

Cell Lines. The BRK-p53val135-E1A cell line has been described (23) and was maintained at 38.5°C in DMEM supplemented with 10% FCS. BRK-p53val135-E1A cells stably expressing *bcl-2* family genes were generated by transfection of various pRcCMV or pcDNA3-based expression plasmids and selection with G418 (250 μg/ml). DNA transfections were carried out by the standard calcium phosphate method.

Cell Death Assays. BRK-p53val135-E1A cells were plated at 5×10^5 cells/60-mm dish. After 24 h at 38.5°C, the dishes were shifted to 32.5°C. At various intervals, cells were trypsinized in triplicates and stained with 0.2% trypan blue; then viable cells were counted.

Immunoprecipitation. Cells were metabolically labeled with 500 μCi of [^{35}S]methionine and [^{35}S]cysteine mixture (DuPont NEN) for 2 h and lysed in isotonic buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS] containing protease inhibitors (0.04 mg/ml aprotinin, 0.2 mg/ml leupeptin, and 200 μM phenylmethylsulfonyl fluoride).

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[4] The abbreviations used are: HA, hemagglutinin; BRK, baby rat kidney.

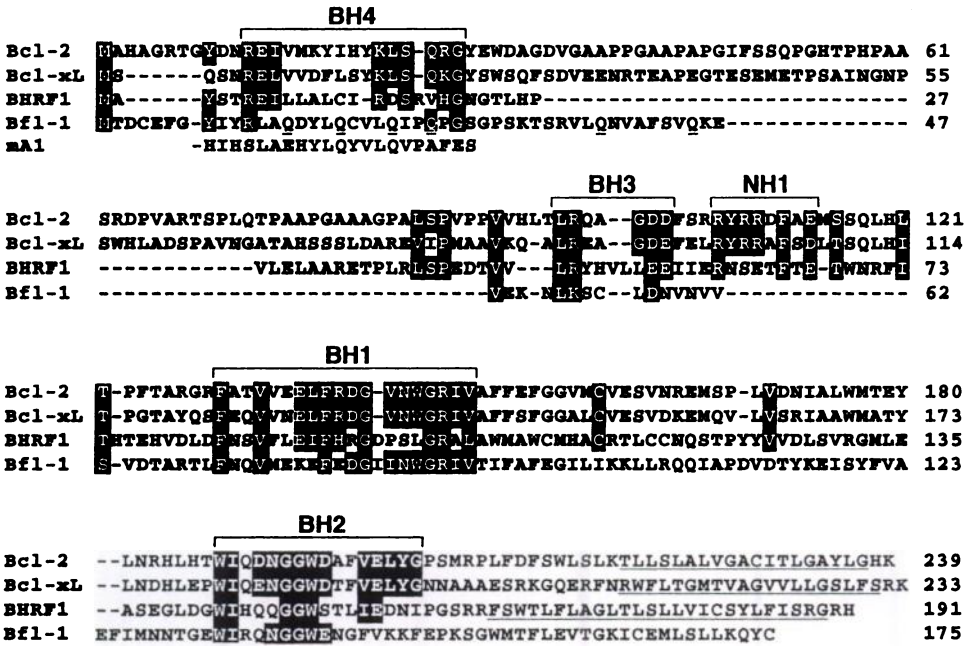

Fig. 1. Amino acid sequence alignment of anti-apoptotic Bcl-2 family proteins. The various conserved domains (BH1-4) are indicated. The Gln residues at the NH_2-terminal region of Bfl-1 are *underlined*. The COOH-terminal *trans*-membrane domains are *underlined*. The region in mouse A1 (mA1) protein corresponding to the BH4 domain is shown.

Lysates (clarified by 30 min microcentrifugation) were precleared with protein A-Sepharose for 1 h, which was removed by centrifugation. The proteins were immunoprecipitated with an anti-HA monoclonal antibody (12CA5; Boehringer Mannheim). The proteins were analyzed by electrophoresis on 15% SDS polyacrylamide gels and detected by fluorography.

Immunofluorescence. Cells were fixed in 3.7% formaldehyde and permeabilized in ice-cold methanol. The cells were then stained with anti-HA monoclonal or anti-Bcl-2 monoclonal antibody (6C8; PharMingen) and counterstained with fluorescein-conjugated antimouse antibody (Pierce) or fluorescein-conjugated antihamster antibody (Boehringer Mannheim).

Results and Discussion

Suppression of p53-induced Apoptosis. To determine if *bfl-1* possesses an anti-apoptosis activity, we used a sensitive assay using cells that express a temperature-sensitive mutant of the p53 tumor suppressor protein (28). BRK cells transformed with adenovirus *E1a* and a *ts* mutant of p53 (p53val135) express very high levels of the mutant p53 protein at the nonpermissive (38.5°C) temperature and undergo rapid apoptosis after the p53 protein assumes *wt* conformation at 32.5°C (29). We and others have previously used this assay system to analyze the anti-apoptosis activity of various Bcl-2 family proteins (23, 25, 30). BRK-p53val135-E1A cells were transfected with the pcDNA3 vector or any of the plasmids expressing Bfl-1, Bcl-2, Bcl-x_L or BHRF1, and G418 (*neo*)-resistant colonies were selected at 38.5°C. A comparable number of colonies were pooled and assayed for survival at 32.5°C. When Bfl-1-expressing cells were shifted to 32.5°C, about 60% of the cells retained viability over a 72-h assay period (Fig. 2). In contrast, cells transfected with the vector exhibited a near total loss of viability within 48 h. Among the other Bcl-2 family proteins, Bcl-x_L conferred the highest survival advantage, resulting in near total cell survival. These cells even exhibited some proliferation. Cells expressing either Bcl-2, BHRF1, or E1B 19 kDa retained about 60–80% viability. These results indicate that the Bfl-1 protein suppresses p53-induced apoptosis at levels comparable to that of Bcl-2, BHRF1, and E1B-19 kDa. All of these proteins were less efficient than Bcl-x_L in suppressing p53-induced apoptosis.

Cooperative Transformation. Since *bfl-1* was found to be overexpressed in gastrointestinal cancers, we decided to determine if it has any dominant transforming activity in primary epithelial cells. We used the well-studied primary rat kidney (BRK) cell system for this purpose since it is considered to be an excellent model system to study oncogenic transformation of primary epithelial cells. To determine if *bfl-1* has an autonomous transforming activity, we transfected BRK cells prepared from 2-day-old neonatal rats either with pcDNA3 or pcDNA3HA-Bfl-1. Transformed colonies were detected by *neo* (G418) selection. In these studies, *bfl-1* did not induce any detectable foci formation. Similarly, other *bcl-2* family members (*bcl-2*, *bcl-x_L*, and *BHRF1*) also did not induce any foci formation (data not shown). We then examined *bfl-1* for cooperative transformation activity with the *E1a* oncogene. The *E1a* oncogene is a well-studied dominant

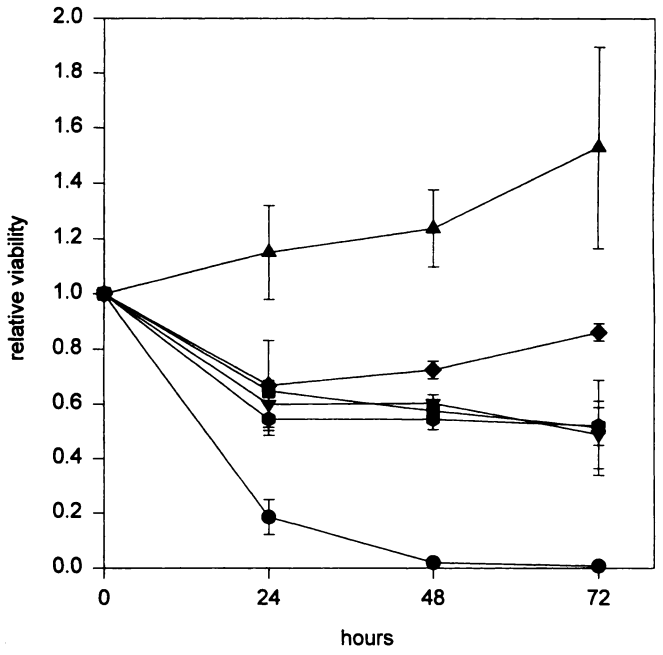

Fig. 2. Suppression of p53-induced apoptosis by *bcl-2* family genes. BRK-p53val135-E1A cells were transfected with various plasmids, *neo*-resistant colonies were pooled, and survival at 32.5°C was determined. ●, pcDNA3; ▲, *bcl-x_L*; ♦, E1B *19K*; ■, *bcl-2*; ●, *bfl-1*; ▼, *BHRF1*.

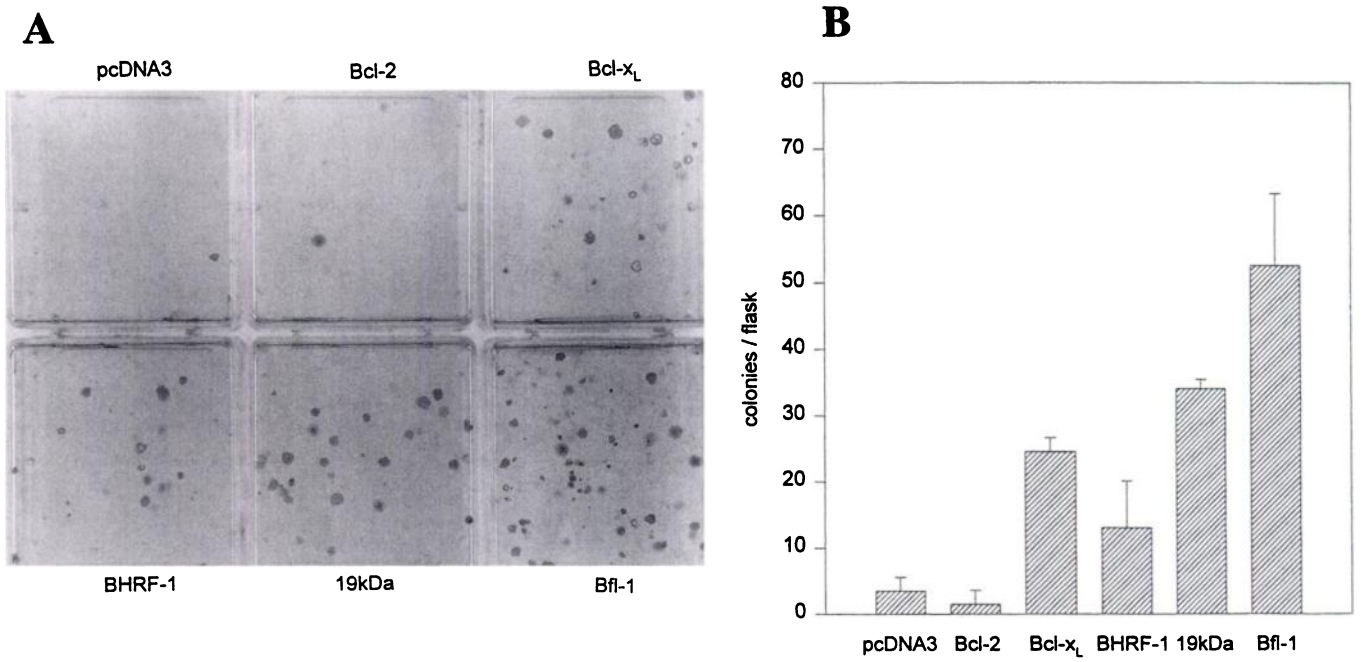

Fig. 3. Cooperative transformation of BRK cells with *E1a* and *bcl-2* family genes. Two μg of E1A and 1 μg of pcDNA3 or plasmids expressing the various *bcl-2* family genes were cotransfected into BRK cells and were selected with G418 (50 μg/ml). Fourteen days after transfection, cells were stained with Giemsa, photographed (*A*) and counted (*B*).

oncogene and exerts its transforming activity by modulating two converging pathways that involve two cellular proteins, p300 and pRb (reviewed in Ref. 31). At least some aspect of E1A-mediated transformation may be ultimately linked to the cellular response to p53 (32). Cells transfected with pE1A and pcDNA-3 vector produced a few slowly growing colonies. In contrast, cells cotransfected with pcDNA3HA-Bfl-1 resulted in a number of rapidly growing foci (Fig. 3*A*). These foci were readily detected by visual examination within a week after transfection. We routinely observe similar efficiency of transformation in BRK cells transfected with *E1a* and the activated *ras* oncogene (data not shown). In contrast to *bfl-1*, transfection of *bcl-2* did not significantly increase the frequency of colony formation compared to cells transfected with *E1a*. Cells cotransfected with *E1a* and either *BHRF1*, *bcl-x*$_L$, or *E1B-19K* produced significant numbers of transformed colonies, albeit at lower levels than *bfl-1*. These results indicate that among the four anti-apoptosis family members we have examined, *bfl-1* has the most potent cooperative transforming activity. It is very interesting to note that *bcl-x*$_L$, despite having a potent anti-apoptosis activity, exhibits a less pronounced cooperative transforming activity when compared to *bfl-1* (Fig. 3*B*).

Subcellular Localization. A characteristic feature of many Bcl-2 family proteins is the presence of a COOH-terminal *trans*-membrane domain consisting of about 20 hydrophobic residues bounded by one or more charged residues. Domain exchange studies have shown that the COOH-terminal hydrophobic domain contributes to the membrane localization of Bcl-2 (33). Unlike the *trans*-membrane domain of other Bcl-2 family proteins, the COOH-terminal hydrophobic domain of Bfl-1 contains three charged residues in addition to the hydrophobic residues. It is, therefore, of interest to determine if Bfl-1 localizes to the same subcellular compartments as Bcl-2. To determine the subcellular localization of Bfl-1, we analyzed BRK-p53val135-E1A or BRK-p53val135-E1A/Bfl-1 cells by indirect immunofluorescence using anti-HA monoclonal antibody. For comparison, BRK-p53val135-E1A/Bcl-2 cells were also examined using anti-Bcl-2 monoclonal antibody. The immunofluorescence analysis revealed that the Bfl-1 protein is predominantly localized in the endoplasmic reticulum/nuclear envelope regions like Bcl-2 (Fig. 4*A*). Immunoprecipitation of HA-tagged protein from BRK-p53val135-E1A/Bfl-1 cells revealed the presence of the expected M_r ~20,000 Bfl-1 protein (Fig. 4*B*). These results indicate the Bfl-1 localizes in the subcellular membrane components in a fashion resembling Bcl-2, despite the absence of a typical *trans*-membrane domain.

In this communication, we have demonstrated that the human *bfl-1* gene suppresses apoptosis induced by high levels of expression of the tumor suppressor protein p53. These results suggest that Bfl-1 may play a role in oncogenesis and resistance to anti-neoplastic treatments by modulating the p53 activity in a manner similar to Bcl-2 [reviewed by Reed *et al.* (34)]. However, it remains to be determined if both Bcl-2 and Bfl-1 mediate their anti-apoptosis activity by similar mechanisms. The amino acid sequence variations at the NH_2 terminus (*e.g.*,

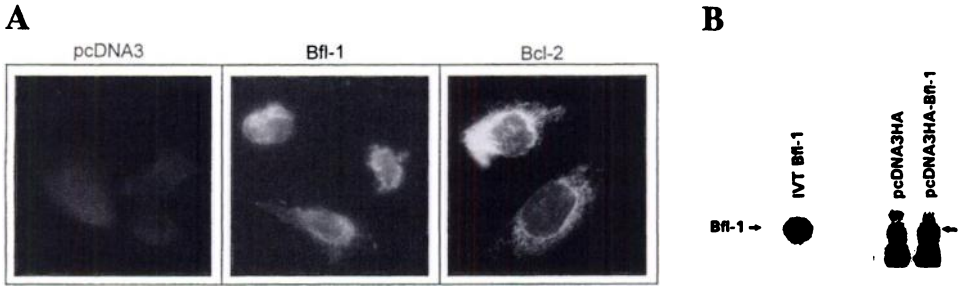

Fig. 4. Immunofluorescence of Bfl-1 (*A*) and immunoprecipitation (*B*). The indirect immunofluorescence analysis was carried out using the anti-HA antibody (with BRK-p53val135-E1A or BRK-p53val135-E1A/Bfl-1 cells) or using the anti Bcl-2 antibody (with BRK-p53val135-E1A/Bcl-2 cells). ^{35}S-Labeled proteins were immunoprecipitated with anti-HA mouse monoclonal antibody and analyzed on a 15% SDS-polyacrylamide gel. IVT-Bfl-1 indicates Bfl-1 protein generated by *in vitro* transcription and translation. *Arrow*, position of Bfl-1 (M_r ~20,000).

presence of the Gln-rich region and the lack of a well-defined BH4 domain) and the apparent lack of the NH1 domain may contribute to the differential activity of Bfl-1. An exciting aspect of our studies is that the *bfl-1* gene exhibits a potent epithelial cell transforming activity in cooperation with the *E1a* oncogene. This observation has important implications in the possible involvement of Bfl-1 in human neoplasia that are of predominantly epithelial cell origin. It is possible that the *bfl-1* gene may cooperate with cellular oncogenes that are functionally analogous to *E1a* (*e.g.*, c-*myc*) in the origin of human neoplastic pathologies. Our results also raise an important issue of fundamental importance concerning the role of *bcl-2* family genes in oncogenesis. Among the various *bcl-2* family genes, only *bcl-2* has thus far been examined in detail for *in vitro* transforming activities. Since *bcl-2* does not exhibit dominant transforming activities in these assays, it is believed that *bcl-2* contributes to oncogenesis by merely suppressing apoptosis (reviewed in Refs. 35 and 36). In our studies, despite comparable anti-apoptosis activity, Bcl-2 does not exhibit significant transforming activity. Similarly, despite potent anti-apoptosis activity, *bcl-*x_L exhibits a lower transformation efficiency than *bfl-1*. These results suggest that an additional activity is required to overcome the "crisis" during transformation of primary cells, and Bfl-1 may be more efficient in the establishment of a transformed state by overcoming the crisis period. It remains to be determined if this activity is linked to the anti-apoptosis activity of these proteins. Detailed mutagenesis of the *bfl-1* gene would be required to dissect the anti-apoptosis and oncogene-cooperating activities to resolve this issue.

Note Added in Proof

In contrast to the *bcl-2* clone used in this study, *wt bcl-2* [Tsujimoto *et al.*, Science (Washington, DC), *228:* 1440–1443, 1985] induces low levels of E1A-cooperative transformation.

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