The Bcl-2 family proteins comprise pro-apoptotic as well as anti-apoptotic members. Heterodimerization between members of the Bcl-2 family proteins is a key event in the regulation of apoptosis. We report here that Bcl-2 protein was selectively cleaved by active caspase-3-like proteases in CTLL-2 cell apoptosis in response to interleukin-2 deprivation. Structural and functional analyses of the cleaved fragment revealed that the NH2-terminal region of Bcl-2 (1–34 amid acids) was required for its anti-apoptotic activity and heterodimerization with pro-apoptotic Bax protein. Site-directed mutagenesis of the NH2-terminal region showed that substitutions of hydrophobic residues of BH4 domain resulted in the loss of ability to form a heterodimer with Bax. Particularly instructive was that the V15E mutant of Bcl-2, which completely lost the ability to form a heterodimer with Bax, failed to inhibit Bax- and staurosporine-induced apoptosis. Our results suggest that the BH4 domain of Bcl-2 is critical for its heterodimerization with Bax and for exhibiting anti-apoptotic activity. Therefore, agents interfering with the critical residues of the BH4 domain may provide a new strategy in cancer therapy by impairing Bcl-2 function.

Apoptosis is essential for development and maintenance of tissue homeostasis and for elimination of harmful cells in multicellular organisms. The Bcl-2 family proteins play an important role in the apoptotic pathway through regulation of the activity of caspasases, which cleave vital cellular substrates to execute apoptosis (2). The Bcl-2 family possesses both pro-apoptotic (Bax, Bak, Bcl-XS, Bad, Bik, Bid, Hrk, and Bim) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1) molecules (3, 4). The ratio of anti-apoptotic to pro-apoptotic molecules, such as Bcl-2/Bax, determines the cell response to an apoptotic signal (5). A striking characteristic of Bcl-2 family members is their propensity to form homodimers with themselves and heterodimers between pro- and anti-apoptotic family members (6, 7).

The Bcl-2 family members have homology clustered within four conserved Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 (4), in which only anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, bear the NH2-terminal BH4 domain. Deletion of either BH1 or BH2 nullifies the ability of Bcl-2 to heterodimerize with Bax and suppress cell death (8). The BH3 domain of pro-apoptotic molecules such as Bax is required for its homodimerization, heterodimerization with Bcl-2 and Bcl-XL, and induction of apoptosis (9, 10). The hydrophobic residues within the BH3 domain of Bax have been found to be critical for dimerization and apoptosis induction (11). In contrast, deletion of the BH3 domain of Bcl-2 did not alter its anti-apoptotic function (12). Structural studies of a Bcl-XL monomer revealed that the BH1–4 domains corresponded to helices 1 to 7. Notably, the BH1, 2, and 3 domains are in close proximity and create a hydrophobic pocket that is presumably involved in interactions with BH3-containing peptides of the death promoters (13, 14). The BH4 domain is also indispensable for its ability to suppress cell death. However, the mechanism of the anti-apoptotic function of the BH4 domain is yet uncertain. It may be involved in regulation of apoptosis by interacting with Raf-1 kinase, calcineurin, and CED-4, resulting in phosphorylation of Bad, impairment of NF-AT signaling, and inhibition of caspase activation, respectively (15–17).

Previous reports, together with our results, showed that Bcl-2 was cleaved by caspase-3-like proteases at D31AGD34, removing the NH2-terminal 1–34 amino acids, which contains the BH4 domain. In the course of analyzing the ΔN mutant of Bcl-2, which mimicked the cleaved Bcl-2 fragment, we found that the NH2-terminal region of Bcl-2 was also required for heterodimerization with Bax and for its anti-apoptotic activity. Furthermore, site-directed mutagenesis identified that the hydrophobic residues in the BH4 domain of Bcl-2 were critical for dimerization with Bax and inhibition of cell death.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Murine IL-2-dependent CTLL-2 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml kanamycin, and 20 units/ml recombinant mouse IL-2. Human embryonic kidney 293T cells and human fibrosarcoma HT1080 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml kanamycin. All cells were maintained at 37 °C in a 5% CO2 and 95% air.

Plasmid Constructions—The pFLAG-CMV-2 vectors (Eastman Kodak Co.) containing murine wild-type bcl-XL (WT-bcl-XL) and the NH2-terminal truncation of bcl-XL (ΔN-bcl-XL, lacking the NH2-terminal 1–61 amino acids) were previously described (18). Murine wild-type bcl-2 (WT-bcl-2), the NH2-terminal truncation of bcl-2 (ΔN-bcl-2, lacking the NH2-terminal 1–34 amino acids), and bax cDNAs were generously provided by Dr. Tomoyoshi Tsuchiya (National Cancer Center Research Institute, Tokyo, Japan and University of Tokyo, Japan) and by Dr. Miki Hirotani (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). The 5′-end of the bcl-XL and bcl-2 cDNAs were digested by BamHI and XhoI, and the products were subcloned into the shuttle vector pEGFP-N1 (Clontech Laboratories, Inc.). The correct orientation of the inserts was confirmed by enzyme digestion and direct sequencing.

This paper is available on line at http://www.jbc.org
ated by reverse transcription-polymerase chain reaction with CTLL-2 mRNA as the template. The polymerase chain reaction products were cloned into a pCRII vector (Invitrogen, San Diego) and subcloned in-frame into an EcoRI site of pc3XP vector, a pcDNA3 vector (Invitrogen) containing an Xpress epitope at the 5’ end, or the pFLAG-CMV-2 vector (Kodak). The pc3XP vector containing wild-type bcl-2 cDNA or the pFLAG-CMV-2 vector containing wild-type bcl-2 cDNA was employed as the template for mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) Substitutions of amino acids (V15E, Y21D, S24E, W30S, D31A, D34A, and D36A) in bcl-2 cDNA were accomplished by converting the corresponding amino acid codon GTG, TTA, TCG, GAT, CAT, or GAC to GAG, GAT, GAA, TCG, GCT, GGT, or GCC, respectively. The proper construction of all plasmids was confirmed by DNA sequencing.

In Vitro Cleavage Assay—The wild-type mouse Bcl-2 and mutant proteins were labeled with [35S]methionine using in vitro transcription/translation system (Promega, Madison, WI). The translated proteins in reticulocyte lysate (2 μl) were incubated with active caspase-3, -6, or -7 in caspase assay buffer for 12 h at 37 °C (18). The reactions were applied to a 15–25% gradient polyacrylamide gel, followed by autoradiography.

Transient Transfection—Experiments were performed as described previously (18). Briefly, 293T cells or HT1080 cells were grown to about 80% confluence in 60-mm plates before transfection. Five μg of the pFLAG-CMV-2 vectors containing mock, WT-bcl-2, ΔN-bcl-2, WT-bcl-ΔX1, ΔN-bcl-ΔX2, or bcl-2 mutant cDNAs were transiently transfected into cells using a Superfect transfection reagent according to the manufacturer’s instruction (Qiagen, Hilden, Germany). The transfected cells were lysed 24 h later, and the cell lysates were used for Western blot analysis and co-immunoprecipitation assay. To investigate the effects of Bcl-2 mutants on the inhibition of Bax-induced apoptosis, each Bcl-2 mutant plasmid (1 μg) was co-transfected into 293T cells with 1 μg of pc5XP-Bax and 1 μg of pcDNA3 vector expressing enhanced GFP protein. To investigate the effects of Bcl-2 mutants on the inhibition of STS-induced apoptosis, each Bcl-2 mutant plasmid (1.5 μg) was co-transfected into HT1080 cells with 1 μg of pcDNA3 vector expressing enhanced GFP protein. The proportion of apoptotic cells were determined in GFP-positive cells that showed nuclear condensation and fragmentation confirmed by DAPI staining.

Measurement of Caspase Activity—293T cells co-transfected with Bax and Bcl-2 mutants were harvested and lysed in lysis buffer containing 10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol. The cell lysates were obtained by centrifugation at 4 °C for 30 min at 40,000 rpm. Twenty μg of the cell lysate were used to incubate with 20 μM fluorogenic substrate DEVD-pNA (Peptide Institute, Osaka, Japan) in caspase assay buffer (20 mM HEPES (pH 7.4), 10% glycerol, and 2 mM dithiothreitol) for 1 h at 37 °C. The AMC released from the fluorogenic substrates was excited at 380 nm, and the emission was measured at 460 nm using a Hitachi fluorescence spectrophotometer, model F-2000 (Hitachi, Tokyo, Japan).

Western Blot Analysis—Cells were solubilized under reduced conditions with lysis buffer containing 0.5% Nonidet P-40. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with an anti-mouse Bcl-2 mAb (Pharmingen, San Diego, CA), an anti-human Bcl-2 pAb (Calbiochem), an anti-human Bax pAb (Upstate Biotechnology, Lake Placid, NY), and an anti-FLAG M5 mAb (Kodak). The membranes were then incubated with peroxidase-conjugated second antibody followed by developing with an enhanced chemiluminescence (ECL) mixture (Amersham Pharmacia Biotech).

Results

Bcl-2 Cleavage during CTLL-2 Cell Apoptosis—We previously reported that IL-2 deprivation could induce apoptosis in IL-2-dependent CTLL-2 cells (18). When we examined the expression of Bcl-2, we found an additional band (23 kDa) cross-reactive to the specific anti-mouse Bcl-2 mAb was observed in apoptotic CTLL-2 cells cultured in medium containing 0 or 0.01 ng/ml IL-2 but not in viable CTLL-2 cells cultured in medium containing 0.1 or 1 ng/ml IL-2 (Fig. 1A). Because caspases were activated during CTLL-2 cell apoptosis, we examined whether this novel 23-kDa band was a cleaved product of Bcl-2. The addition of the cell-permeable caspase inhibitor Z-VAD, Z-EVD, or Z-Asp to CTLL-2 cells cultured in IL-2-depleted medium suppressed the activation of caspase-3-like proteases (data not shown) and blocked the appearance of the 23-kDa band (Fig. 1B), indicating that the novel 23-kDa protein was indeed a cleaved Bcl-2 fragment.

To identify the still unclear cleavage sites in mouse Bcl-2 protein, we constructed several Bcl-2 mutant cDNAs in which the aspartic acid codon at 31, 34, or 36 was converted to the alanine codon (D31A, D34A, or D36A, respectively) because the caspase-3-like proteases specifically cleaved its substrates at DXXD motif (in which X may be any amino acid). The wild-type and mutant Bcl-2 proteins were produced by in vitro transcription/translation in reticulocyte lysates and were incubated with recombinant active human caspase-3, -6, or -7. As shown in Fig. 1C, the wild-type Bcl-2 was cleaved by caspase-3, -6, and -7, whereas the D31A and D34A mutants, but not the D36A mutant, could block the cleavage by caspase-3. This suggested that Bcl-2 protein was cleaved at the site D13AGD34 | A. Similar to Bcl-2, Bcl-XL was also cleaved by caspase-3 at the site of HLA-A9 | S and SSSL7 | A (18). Both Bcl-2 and Bcl-XL lost the NHL-terminal region containing the BH4 domain, which is only conserved in anti-apoptotic Bcl-2 family proteins.
Bcl-2 alone could not induce apoptosis in 293T cells (data not shown).

Requirement of the NH$_2$-terminal Region of Bcl-2 for Heterodimerization with Bax—To further investigate the molecular mechanisms by which ΔN-Bcl-2 lost its apoptosis inhibitory function, we examined its ability to bind to Bax in vivo. The binding ability of ΔN-Bcl-X$_L$ to Bax was also investigated. Sequential immunoprecipitation and Western blot analysis were performed using cell extracts from the transfected 293T cells expressing FLAG-tagged wild-type and ΔN-mutant constructs of Bcl-2 and Bcl-X$_L$ to determine whether deletion of the NH$_2$-terminal region disrupted their heterodimerization with endogenous Bax. After transfection for 24 h, the size and stability of the corresponding proteins were confirmed by examining extracts from the transfected cells by Western blot analysis using an anti-FLAG M5 mAb (Fig. 3A, upper panel). The extracts from each transfectant had comparable amounts of Bax protein before immunoprecipitation experiments, as confirmed by Western blot analysis using an anti-human Bax pAb (Fig. 3A, lower panel). The binding of ΔN-Bcl-X$_L$ to Bax was also investigated. Sequential immunoprecipitation and Western blot analysis were performed using cell extracts from the transfected 293T cells expressing FLAG-tagged wild-type and ΔN-mutant constructs of Bcl-2 and Bcl-X$_L$ to determine whether deletion of the NH$_2$-terminal region disrupted their heterodimerization with endogenous Bax. After transfection for 24 h, the size and stability of the corresponding proteins were confirmed by examining extracts from the transfected cells by Western blot analysis using an anti-FLAG M5 mAb (Fig. 3A, upper panel). The extracts from each transfectant had comparable amounts of Bax protein before immunoprecipitation experiments, as confirmed by Western blot analysis using an anti-human Bax pAb (Fig. 3A, lower panel). When these FLAG-tagged proteins were immunoprecipitated with an anti-FLAG M2 mAb and then subjected to Western blot analysis using an anti-Bax pAb, both wild-type Bcl-2 and Bcl-X$_L$ proteins bound to Bax. In contrast, the NH$_2$-terminal-deleted mutants of Bcl-2 and Bcl-X$_L$ failed to interact with Bax (Fig. 3B). These results indicate that the NH$_2$-terminal regions of Bcl-2 (amino acids 1–34) and Bcl-X$_L$ (amino acids 1–61) are necessary for heterodimerization with Bax.

Identification of Residues Critical for Heterodimerization with Bax—The NH$_2$-terminal regions of Bcl-2 and Bcl-X$_L$ contain the BH4 domain that is only conserved in the anti-apoptotic Bcl-2 family proteins (Fig. 4A). To investigate the specific, conserved sequence of the BH4 domain in the NH$_2$-terminal region that is critical for the ability of Bcl-2 to form a heterodimer with Bax, we generated four site-directed point mutants in which the most conserved hydrophobic residues (Val$_{15}$, Tyr$_{21}$, and Trp$_{30}$) or the Raf-1 kinase target residue (Ser$_{24}$) (13) were

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**Fig. 2.** NH$_2$-terminal deletion of Bcl-2 fails to protect 293T cells from Bax-induced caspase activation and apoptosis. A, schematic of the cleavage site of caspase in Bcl-2 protein and the NH$_2$-terminal deletion mutant of Bcl-2, which mimics the cleaved Bcl-2 fragment. B and C, 293T cells were co-transfected with pcDNA3 vector encoding WT-bcl-2 (WT) and ΔN-bcl-2 (ΔN) in the presence of pcDNA3 vector encoding GFP cDNA. After transfection for 8 h, the caspase-3-like protease (DEVDase) activity in the cell lysates was determined by-incubating with DEVD-AMC, as described under “Experimental Procedures.” The basal DEVDase activity in the mock transfectant (solid column) was normalized as 100% (B); the numbers of apoptotic cells were determined by counting cells showing nuclear condensation and fragmentation confirmed by DAPI staining in 100 GFP-positive (transfected) cells (C). Each point represents a mean ± S.D. of triplicate determinations.

**Fig. 3.** NH$_2$-terminal deletion of Bcl-2 and Bcl-X$_L$ abrogates the ability to heterodimerize with Bax. A, 293T cells were transiently transfected with either pFLAG-CMV vector alone or pFLAG-CMV vectors encoding WT-bcl-X$_L$, ΔN-bcl-X$_L$, WT-bcl-2, and ΔN-bcl-2 cDNAs. The transfected 293T cell lysates were subjected to SDS-PAGE and then analyzed by Western blot (WB) analysis using an anti-FLAG M5 mAb (upper panel) or anti-Bax pAb (lower panel). B, the FLAG-tagged Bcl-X$_L$ and Bcl-2 proteins were immunoprecipitated (IP) using the agarose-conjugated anti-FLAG M2 mAb and then immunoblotted with an anti-Bax pAb. IgG (L) indicates the immunoglobulin light chain. The data shown are representative of three independent experiments.
Thus, the Val15 residue exhibited a critical role in modulating heterodimerization with Bax. A, sequence comparison of BH4 domain among anti-apoptotic Bcl-2 family members. **Bold column** indicates residues that form α-helix. Four conserved residues in the BH4 domain (Val15, Tyr34, Ser36, and Trp85) which were mutated in this study are shaded. B, effect of mutations of BH4 domain in Bcl-2 on the binding to Bax. 293T cells were transiently transfected with pFLAG-CMV vector alone (Mock) or pFLAG-CMV vectors encoding WT-bcl-2, ΔN-bcl-2, or the indicated BH4 point mutants. The transfected 293T cell lysates were subjected to SDS-PAGE and then analyzed by Western blot (WB) analysis using an anti-Bcl-2 mAb (upper panel) or an anti-Bax pAb (lower panel). C, the FLAG-tagged proteins were immunoprecipitated (IP) from the transfected 293T cell lysates using the agarose-conjugated anti-FLAG M2 mAb and then probed with an anti-Bax pAb. IgG(L) indicates the immunoglobulin light chain. The data shown are representative of three independent experiments.

converted to charged residues, as shown in Fig. 4A. These Bcl-2 mutants were transfected into 293T cells, and we analyzed their ability to bind to Bax in vivo. The transfection of the mutant Bcl-2 proteins did not affect the expression level of endogenous Bax in 293T cells (Fig. 4B, lower panel). Interestingly, the V15E mutant completely abrogated the ability to interact with Bax like ΔN-Bcl-2 did (Fig. 4C, lanes 4 and 3, respectively). Other amino acid replacements, such as Y21D and W30S mutants, also displayed reduced binding ability to Bax (Fig. 4C, lanes 5 and 7, respectively). In contrast, the S24E mutant displayed the same binding capability with Bax as WT-Bcl-2 did (Fig. 4C, lanes 6 and 2, respectively). These results suggest that the hydrophobic surface within BH4 domain is critical for Bcl-2 to interact with Bax.

**BH4 Mutants That Disrupt the Heterodimerization with Bax Failed to Suppress Bax- and STS-induced Apoptosis**—Because Bax has been reported to form homodimers on the mitochondria membrane to initiate caspase activation and apoptosis, we further investigated whether the BH4 mutant (V15E), which lost the ability to bind to Bax, could inhibit caspase activation and apoptosis. Because the overexpression of V15E mutant itself could not induce apoptosis in 293T cells (data not shown), we examined its apoptosis-regulatory effect on BH4 mutants. The pFLAG-CMV-2 vector containing WT-bcl-2, ΔN-bcl-2, or V15E mutant was co-transfected with the pc5XP vector containing wild-type bax cDNA and the pcDNA3 vector containing GFP cDNA into 293T cells. After incubation for 8 h, the apoptotic cells were counted among the GFP-positive cells with nuclear fragmentation confirmed by DAPI staining, as shown in Fig. 5B. Expression of wild-type Bcl-2 inhibited Bax-induced apoptosis. In contrast, both the V15E mutant and ΔN-Bcl-2 failed to protect cells from undergoing Bax-induced apoptosis (Fig. 5A). Furthermore, we examined whether V15E mutant lost the apoptosis-inhibitory function in STS-induced apoptosis. After transfection of the pFLAG-CMV-2 vector containing WT-bcl-2, ΔN-bcl-2, or V15E mutant in HT1080 cells, the transfected cells were treated with 1 μM STS for 8 h. As shown in Fig. 5C, V15E mutant and ΔN-Bcl-2 could not suppress the STS-induced apoptosis in HT1080 cells, whereas wild-type Bcl-2 protected cells from undergoing apoptosis. Thus, the Val15 residue exhibited a critical role in modulating the structure and function of BH4 domain of Bcl-2.

Interestingly, when examining the amount of Bcl-2 protein in transfected 293T cells, we found that the co-transfection of bax cDNA reduced the expression level of exogenous ΔN-Bcl-2 and V15E mutant (Fig. 6, lower panel, lanes 2 and 3), as compared with that of WT-Bcl-2 (Fig. 6, lower panel, lane 1). Similarly, the amount of exogenous Bax, but not the endogenous Bax, was also decreased in ΔN-Bcl-2 and V15E mutant transfectants (Fig. 6, middle panel, lanes 2 and 3). To elucidate the reason for the decreased expression of both Bax and mutant Bcl-2 proteins in the transfected cells, we performed the above co-transfection experiments in the presence of the caspase inhibitor Z-Asp. Z-Asp could recover the exogenous protein level of both ΔN-Bcl-2, V15E mutant (Fig. 6, lower panel, lanes 5 and 6) and Bax (Fig. 6, middle panel, lanes 5 and 6). However, the cysteine protease inhibitor Z-LLH and calpain inhibitor E-64 had no such effects (data not shown). These data suggested that the reduced expression of exogenous ΔN-Bcl-2, V15E mutant, or Bax was caused by rapid cell death in the ΔN-Bcl-2 and V15E mutant-transfected cells because of the inability of ΔN-Bcl-2 and V15E mutant to suppress Bax-induced apoptosis, as shown in Fig. 5. To rule out that the reduced binding abilities of ΔN-Bcl-2 and V15E mutant with Bax are not due to the decreased expression of these proteins in 293T cells, we tested the abilities of ΔN-Bcl-2 and V15E mutant to form heterodimers with Bax in the presence of Z-Asp. As shown in Fig. 6, wild-type Bcl-2 could bind both exogenous Xpress-tagged Bax and endogenous Bcl-2 (upper panel, lanes 1 and 4). In contrast, the ΔN-Bcl-2 and V15E mutant lost their abilities to form heterodimers with Bax even in the presence of Z-Asp (Fig. 6, upper panel, lanes 5 and 6). Taken together, our results provide an instructive example that the V15E mutant could not inhibit apoptosis due to the reduced capacity to dimerize with Bax.

**DISCUSSION**

The Bcl-2 protein plays an essential role in preventing cell death. Its anti-apoptotic activity is regulated through association with Bcl-2 homologous and nonhomologous proteins and also by phosphorylation at the serine residue (12). Site-directed mutagenesis of two BH domains revealed at least three discrete functional regions. The first region (transmembrane domain) is a hydrophobic domain at the COOH terminus that confers membrane anchorage; deletion of this region reduces but does not eliminate Bcl-2 activity (19). The second region (BH1 and BH2 domains) is critical for both their anti-apoptotic function and the capacity to heterodimerize with pro-apoptotic Bcl-2 family proteins like Bak or Bax (7, 8, 20). Almost all BH1 or BH2 mutants that disrupt heterodimerization with Bax lose...
panels.

**Fig. 5.** V15E mutant of Bcl-2 abrogates the ability to inhibit apoptosis. A, 293T cells were co-transfected with pc5XP-Bax and pFLAG-CMV vectors encoding WT-bcl-2, ΔN-bcl-2, or V15E mutant in the presence of pcDNA3 vector encoding GFP cDNA. After transfection for 8 h, the numbers of apoptotic cells were determined by counting cells showing nuclear condensation and fragmentation and confirmed by DAPI staining in 100 GFP-positive (transfected) cells. Each point represents a mean ± S.D. of triplicate determinations. B, representative views of the above-transfected 293T cells stained with DAPI (lower panels) and viewed by immunofluorescence microscopy (GFP; upper panels). C, HT1080 cells were transfected with pFLAG-CMV vectors encoding WT-bcl-2, ΔN-bcl-2, or V15E mutant in the presence of pcDNA3 vector encoding GFP cDNA. After transfection for 24 h, the cells were treated with 1 μM staurosporine for 8 h, and the numbers of apoptotic cells were determined by DAPI staining in 100 GFP-positive cells.

Bcl-2 family contain only the BH3 domain but not BH1, BH2, or BH4 (30). In contrast, mutations within the BH3 domain do not significantly affect the anti-apoptotic functions of Bcl-2. Consistent with this, a genetic approach with Bcl-2-deficient and Bax-deficient mice also suggested that Bax could function independently of Bcl-2 (31). Thus, Bax seems to dominantly induce apoptosis by its own activity rather than by blocking the anti-apoptotic activity of Bcl-2 or Bcl-XL, whereas Bcl-2 and Bcl-XL inversely inhibit apoptosis through forming heterodimers with Bax or other types of proteins ranging from protein kinases and phosphatases to proteins that bind caspases (15–17, 20).

Previous results and the present report showed that the cleavage of Bcl-2 (both in human and mouse Bcl-2) by caspases (Fig. 1) (22, 32) removed the NH2-terminal region of Bcl-2 (amino acid 1–34), which contains the BH4 domain that is conserved among the anti-apoptotic Bcl-2 family members. Our data revealed that the NH2-terminal deletion mutant (ΔN-Bcl-2), which mimics the cleaved Bcl-2 fragment, could not suppress Bax- and STS-induced apoptosis in transfected 293T cells and HT1080 cells, respectively (Figs. 2 and 5). Although the NH2-terminal region is critical for anti-apoptotic Bcl-2 family proteins to prevent cells from undergoing apoptosis, the molecular mechanisms by which loss of NH2-terminal region affect the activity of Bcl-2 has not been fully understood. Our data reported herein showed that deletion of the NH2-terminal region of Bcl-2 and Bcl-XL abrogated their heterodimerization with Bax (Fig. 3B) in mammalian cells in vivo and thus lost their apoptosis-inhibitory activity, suggesting that the heterodimerization of Bcl-2 with Bax depends on not only the BH1–2 but also BH4 domains. This is consistent with the previous reports showed a requirement for NH2-terminal region of Bcl-2 for binding to Bax (6) and Bad (33) in a yeast two-hybrid system.

The structural analysis of the Bcl-XL monomer promoted a molecular model of the BH4 domain within the NH2-terminal region of Bcl-2, which revealed an amphipathic helix (α1 helix) on the surface that forms extensive hydrophobic interactions with α2, α5, and α6 (13). Through mutagenesis analysis of the BH4 domain in Bcl-2, we revealed several critical amino acids responsible for modulating the conformation of BH4 domain.

**Fig. 6.** Failure of ΔN-Bcl-2 and V15E mutant to interact with Bax is not due to the instability of transfected proteins. 293T cells were transiently co-transfected with pc5XP-Bax and pFLAG-CMV vectors encoding WT-bcl-2, ΔN-bcl-2, or V15E in the presence or the absence of Z-Asp (100 μg/ml). After transfection for 24 h, the transfected 293T cells were subjected to SDS-PAGE, followed by Western blot (WB) analysis using an anti-Bax M5 mAb (lower panel) or an anti-Bax pAb (middle panel). The FLAG-tagged proteins were immunoprecipitated (IP) using the agarose-conjugated anti-FLAG M2 mAb and then probed with an anti-Bax pAb (upper panel). The asterisk indicates endogenous Bax. Data shown are the representative of three independent experiments.

Growing evidence has demonstrated that Bax may dominantly regulate caspase activation (23). The ability of Bax to localize to mitochondrial membranes (24, 25) and to form homodimers (22) may relate to its ability to form distinct ion-conductive channels (26, 27) and consequently to induce mitochondrial dysfunction, caspase activation, and cell death (11). The structural features of Bax and Bcl-2 that allow them to independently regulate caspase activation (23). The ability of Bax to participate in homo- and heterodimerization phenomena are markedly different, despite their amino acid sequence similarity. Deletional analysis studies indicate that BH3 is a critical domain of Bax for both homo- and heterodimerization (9, 10). This is also consistent with the capacity of Bax-derived BH3 peptides to block homo- and heterodimerization with Bcl-2 family members (28). Bcl-2 deleted Bax molecules also showed impaired killing activity (7, 9, 29). Some cell death activators in the death repressor function. The third region is located at the NH2 terminus. The NH2-terminal deletion mutants not only failed to prevent apoptosis (21) but also functioned as trans-dominant inhibitors of wild-type Bcl-2 (19, 22), although its mechanism is unknown.

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and affecting its function. The V15E mutant, which replaced a hydrophobic residue with a charged residue, proved most instructive because it lost the ability to heterodimerize with Bax (Fig. 4) as well as its anti-apoptotic activity (Fig. 5), consistent with the fact that the ability of V15G mutant of Bcl-2 to inhibit staurosporine-induced apoptosis in fibroblasts was totally abrogated (34). W30S and Y21D mutants also displayed altered capacities to heterodimerize with Bax, although less dramatically than V15E mutant. In contrast, substitution of the serine phosphorylation site S24E displayed a normal pattern of heterodimerization with Bax (Fig. 4). This may rule out the possibility that the altered Bax binding ability of the above point mutations is due to a close location to the phosphorylation site. Taken together, these results suggest that the maintaining the amphipathic nature of the α1 helix in BH4 domain is critical for Bcl-2 to form a heterodimer with Bax and to protect cells from killing. Without hydrophobic surface of BH4, the α1 helix cannot dock on the backside of the Bcl-2 protein and probably the protein alters its conformation so that the hydrophobic pocket, which normally binds the BH3 domain of Bax, is altered. Thus, introducing a charged residue on this hydrophobic face appears to be particularly deleterious to the anti-apoptotic function of Bcl-2. This may indicate that hydrophobic interactions at the base of the binding pocket of Bcl-2 are more important than electrostatic interactions.

These results indicate that the BH4 domain, besides the BH1 and BH2 domains, of Bcl-2 is also essential for heterodimerization with Bax. Our data supported that the interaction of Bcl-2 with Bax is critical for exhibiting its anti-apoptotic activity, whereas we could not rule out that other Bcl-2-interacted proteins may be also involved in BH4 domain function. Our findings provide an opportunity to develop new agents to promote tumor cell apoptosis by interrupting the function of the BH4 domain.

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