Bcl-2 is the best characterized member of a large family of proteins that regulate apoptosis. Although it is established that Bcl-2 localized at the mitochondria functions as an anti-apoptotic protein, the function of Bcl-2 at the nucleus remains unclear. Recently we showed that nuclear compartment-associated Bcl-2 inhibits transcription factor activation. Based on this observation, we hypothesized that presence of Bcl-2 at the nucleus may induce rather than protect cells from apoptosis. Here we investigated the putative apoptotic role of nuclear compartment-associated Bcl-2. Additionally, we examined the role of the Bcl-2 BH4 domain in mediating binding to FKBP38, the Bcl-2 mitochondrial chaperone. Our results demonstrate a novel, pro-apoptotic function for nuclear Bcl-2 and identify the Bcl-2 BH4 domain as a key regulator in mediating Bcl-2/FKBP38 binding. These results indicate that Bcl-2 has a dual role as both a protector and a killer and that the ability to switch roles depends on Bcl-2 subcellular localization.

Programmed cell death, or apoptosis, is a complex and well organized cellular process important during development as well as normal tissue maintenance (1, 2). Abrogation of apoptotic regulation or failure to execute a precise cascade of apoptotic events can lead to premature cell loss or survival of undesired cells (3). Regulation of apoptosis by the Bcl-2 family of proteins has been extensively characterized (4–6). This family of proteins comprises more than 20 members, which can be further divided into 2 broad subfamilies depending on their function as either anti- or pro-apoptotic proteins. The prototypical anti-apoptotic Bcl-2 family member is Bcl-2 itself. Cells stably transfected with Bcl-2 are resistant to several apoptosis-inducing stimuli, including staurosporine, serum withdrawal, and H2O2 treatment (7, 8). On the other hand, the prototypical pro-apoptotic family member is Bax. Overexpression of Bax induces apoptosis and exacerbates the extent of cell death following apoptotic cell stressors (9).

The ability of Bcl-2 to protect cells from apoptosis is critically influenced by protein-protein interactions, post-translational modifications, and notably, subcellular localization (10–12). Bcl-2 primarily localizes to cytoplasmic facing membranes and is most commonly associated with the mitochondria, endoplasmic reticulum (ER), and nuclear envelope (13). Although Bcl-2 localizes to multiple organelles, its anti-apoptotic function has predominantly been investigated at the mitochondria, a site in which Bcl-2 is actively transported to via the mitochondrial chaperone protein FKBP38 (14), an atypical member of the FK506-binding immunophilin protein family (15). FKBP38 binds and shuttles Bcl-2 away from its default localization at the ER and nuclear envelope and places it at the mitochondria (16). Perturbations of this shuttling mechanism both alter the subcellular localization of Bcl-2 and interfere with its anti-apoptotic function (14). Once Bcl-2 is placed on the outer mitochondrial membrane, it is capable of forming heterodimers with pro-apoptotic family members such as Bax and, thus, prevents initiation of apoptosis through blocking the formation of mitochondrial transition pores, release of cytochrome c, and subsequent activation of a caspase-9/caspase-3 cascade (17, 18).

Although FKBP38 is crucial for directing Bcl-2 to the mitochondria, little is known about the mechanisms of interaction between Bcl-2 and FKBP38 (19) or the function of non-mitochondrial localized Bcl-2. Recent reports have described a role for Bcl-2 at the ER in regulating intralumen calcium levels, thus contributing to prevention of apoptosis through buffering of intracellular free calcium (20–22). Although ER-associated Bcl-2 is gaining research interest, the actions of nuclear compartment-associated Bcl-2 remains unclear. Among the limited reports of nuclear Bcl-2 function, two distinct activities have been observed. These include the ability of nuclear compartment-associated Bcl-2 to sequester GSH within the nucleus (23) and the ability to block transcription factor activation (11).

We have previously shown that in aged rats there is an oxidative stress-dependent up-regulation of nuclear compartment-associated Bcl-2 (24, 25). In addition, this nuclear compartment-associated Bcl-2 failed to protect cells from apoptosis induced by oxidative stress (26). More recently, we reported that Bcl-2 localized at the nuclear compartment decreased activity of several transcription factors, particularly nuclear factor κ B (NFκB), likely through depression of nuclear trafficking (11). Given the requirement of proper NFκB activity to prevent cell death both in vitro (27) and

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\[1\] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[The abbreviations used are: ER, endoplasmic reticulum; HEK cells, human embryonic kidney cells; siRNA, small interfering RNA; HA, hemagglutinin; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; IP, immunoprecipitation; PI, propidium iodide; YFP, yellow fluorescent protein.]

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Induction of Apoptosis by Nuclear Bcl-2

in vivo (28), these results suggested that nuclear Bcl-2 may induce rather than prevent apoptosis.

The notion that Bcl-2 can act in a pro-apoptotic fashion, albeit counterintuitive, has been previously reported. Both transient expression of Bcl-2 (29, 30) and caspase 3 cleavage of Bcl-2 (31, 32), an event that removes the Bcl-2 BH4 domain and converts Bcl-2 into a Bax like protein, have been shown to induce apoptosis. Furthermore, interaction of Bcl-2 with the nuclear orphan receptor Nur-77, a protein that leaves the nucleus during apoptosis and binds Bcl-2 at the mitochondrion, also converts Bcl-2 into a pro-apoptotic protein (33).

Based on published evidence of pro-apoptotic Bcl-2 activity and our observation that nuclear compartment-associated Bcl-2 blocks transcription factor activation, we have investigated our observation that nuclear compartment-associated Bcl-2 functions as a pro-apoptotic protein and that localization of Bcl-2 at the nucleus results from failure of FKBP38-mediated delivery of Bcl-2 to the mitochondria. In addition, we show that a Bcl-2 deletion mutant lacking the BH4 domain (Bcl-2ΔBH4) fails to bind FKBP38 and is solely localized at the nucleus, where it promotes apoptosis. Last, we show that an 18-amino acid peptide corresponding to the BH4 domain of human Bcl-2 binds to FKBP38. Collectively, these results illustrate that Bcl-2 can act as both a pro- or anti-apoptotic regulator depending on its subcellular localization and that the Bcl-2 BH4 domain is critical for regulating Bcl-2 subcellular localization via binding to FKBP38. These findings suggest a target for future utilization of endogenous Bcl-2 as a specific cell death inducer through the modulation of the mechanisms controlling Bcl-2 subcellular localization and function.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

Cell culture and general reagents were obtained from Invitrogen, Fisher, and Sigma-Aldrich unless specified otherwise. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). pMKitNeo (vector) and pMKitNeo-Bcl-2a were kind gifts of Dr. S. May (University of Florida, Gainesville, FL). pHA-FKBP38 was a kind gift of Dr. K. I. Nakayama (Kyushu University, Fukuoka, Japan). Mito-Bcl-2 and Mito-Bcl-2-GFP were both kind gifts of Dr. C. W. Distelhorst (Case Western Reserve University, Cleveland, OH). Bcl-2ΔBH4 was generated by introducing a start codon at amino acid position 25 of parental pMKitNeo-Bcl-2a flanked by a XhoI site. The obtained fragment was then digested with Xhol/EcoRI and re-cloned into pMKit-Neo. pEYFP-N1 (YFP) vector was purchased from Clontech (Mountain View, CA). pBcl-2/YFP was generated by PCR amplification of amino acids 1–661 of parental pMKitNeo-Bcl-2a, substituting the stop codon of Bcl-2 with an alanine flanked by an XhoI site. The obtained fragment was then digested with Xhol/EcoRI and re-cloned into pEYFP-N1 in-frame with its EYFP coding region.

Cell Culture

Stock cultures of rat pheochromocytoma PC12 (ATCC, Manassas, VA) were maintained in 75-cm² tissue culture flasks in 12 ml of RPMI 1640 culture medium (Invitrogen) supplemented with 5% (v/v) heat-inactivated donor horse serum (Invitrogen), 5% heat-inactivated fetal bovine serum (Gemini, Woodland, CA), and 1% antibiotic mixture penicillin, streptomycin (Cellgro, Herndon, VA) in a humidified cell incubator at 37 °C under a 5% CO₂ atmosphere. B104 cells were maintained in 75-cm² tissue culture flasks in 12 ml of Dulbecco’s modified Eagle’s medium/F-12 culture medium supplemented with 10% fetal bovine serum. Both HeLa and HEK293 cells were maintained in 75-cm² tissue culture flasks in 12 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For all cultured cells medium (5 ml) was replaced every other day for maintenance of cell viability. Cells were subcultured twice a week. PC12 and B104 cells were subcultured by shaking flask, and 25% of cells were removed to a new culture flask and replaced with normal growth medium. Subculturing HeLa and HEK293 cells was achieved by incubating cells for 5 min with PBS supplemented with 5 mM EDTA (pH 8.0) to detach cells from the plate surface. After detachment, cells were collected, centrifuged (800 × g for 5 min), and ultimately plated at ¼th the original cell density in fresh growth medium.

Transfections

All transfections were performed using Lipofectamine with Plus reagent (Invitrogen). Transfections were carried out via the manufacturer’s instructions. Briefly, cells at 40–50% confluence received DNA/plus reagent coupled to Lipofectamine and diluted in Opti-MEM (Invitrogen). 3 h post-transfection, the liposome/DNA mixture was replaced with fresh culture medium, and the transiently transfected cells were allowed 24–48 h of recovery. Stable cell lines were generated using the same transfection method followed by the addition of a selective antibiotic to the culture medium (Geneticin; Invitrogen).

Small Interfering RNA

PC12 cells (1.0 × 10⁵) were seeded into 6-well plates and grown to 50% confluency. The fkbp8 (FKBP38 small interfering RNA (siRNA)) (ON TARGETplus SMARTpool siRNA; Dharmacon, Lafayette, CO) or control siRNA (ON TARGETplus SMARTpool siRNA; Dharmacon) was combined with DharmaFECT transfection reagent #1 (Dharmacon), and the cells were transfected according to the recommended protocol with siRNA (100 ns final concentration). After 48 h of transfection, cell lysates were prepared for Western blot analysis.

Peptide Synthesis

Peptides were generated by solid-phase peptide synthesis using standard Fmoc chemistry and purified by HPLC. Identity was confirmed by amino acid analysis and mass spectrometry. hBcl-2 amino acids 11–28, BH4 (DNREIVMKYIHYKLQSRG) and BH4scramble (VKRDYRSMIKGEHQLYIN) peptides were synthesized by the University of Texas Medical Branch protein synthesis core and were HPLC-purified to >95% purity.

Western Blotting

Western blotting was performed as previously described (11). Briefly, protein extracts were resolved on a 12% SDS-polyacrylamide denaturing gel, transferred to a nitrocellulose membrane, blocked, incubated with primary antibody, washed, incubated with a horseradish peroxidase-conjugated secondary antibody,
and washed, and immunoreactive bands were detected using a chemiluminescent Western blot detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Antibodies**

Both Pan-Lamin and FKBPNI1 were generous gifts from Dr. Allan Fields (Mayo Clinic, Jacksonville) and Dr. K. I. Nakayama (Kyushu University, Fukuoka, Japan), respectively; all other antibodies were purchased from sources as indicated: monoclonal HA antibody 12CA5 (Active Motif, Carlsbad, CA), monoclonal Bcl-2 10C4, polyclonal Bcl-2 N19, polyclonal IκBα (FL) (secondary 1:2500) (Santa Cruz, Santa Cruz, CA), polyclonal Bcl-2 554087 (BD Pharmingen), calnexin polyclonal antibody (Clontech, Mountain View, CA), monoclonal Bcl-2 554087 (BD Pharmingen), calnexin polyclonal antibody (Clontech, Mountain View, CA), monoclonal β-actin AC-15 (secondary 1:50,000) (Sigma). All horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad and used at 1:7500 unless stated otherwise.

**Cell Fractionation**

Performed as described previously (34). Briefly, for non-nuclear proteins, cells were lysed in hypotonic lysis buffer and centrifuged. The resulting nuclear pellet was further purified as described under nuclear proteins section below. The supernatant was transferred to a new 1.5-ml tube and centrifuged at 1000 \( \times g \) for 10 min at 4 °C. The supernatant from this second centrifugation (non-nuclear protein) was transferred to a new 1.5-ml tube, and the protein concentration in each sample was measured using a bicinchoninic acid protein assay kit according to the manufacturer’s instructions (Pierce). For nuclear proteins, after isolation of the nuclear pellet (as described above), nuclei were lysed in 50 μl of SDS lysis buffer (5 mM EDTA, 50 mM Tris, and 2% SDS) and disrupted by sonication for 15 s. Protein concentration was determined in each sample using the standard bicinchoninic acid protein assay.

**Nuclei Isolation**

Isolation of nuclei was performed as previously described (34). Briefly, cells were lysed in hypotonic lysis buffer, and nuclei were pelleted by centrifugation, resuspended, layered on a 1.8 M sucrose cushion, and ultracentrifuged. Centrifuged nuclear pellets were re-suspended in PBS and centrifuged. Pelleted nuclei were then resuspended in appropriate assay buffer and utilized for further analysis.

**Confocal Microscopy**

Isolated nuclei were resuspended in Vecta-shield hard mount fluorescent mounting medium (Vector Laboratories, Burlingame, CA) and mounted on 1.5 borosilicate round cover glass (Fisher). Mounting medium was allowed to set at room temperature in the dark for 2 h. Slides were then stored at 4 °C in the dark until imaging. Images were obtained using a Zeiss LSM510 META advanced laser scanning confocal microscope with laser excitations at 351, 364, 457, 477, 488, 514, 543, and 633 nm. Differential interference contrast and fluorescent imaging analysis was performed using Meta-morph imaging software V.6.0 (Universal Imaging Corp., Downingtown, PA).

**Induction of Apoptosis by Nuclear Bcl-2**

Cells were plated between 40 and 50% confluency into 12-well plates. After 24 h of recovery cells were transiently transfected. 24–48 h post-transfection these cells were washed 3 times with PBS (pH 7.4) then resuspended in 500 μl of annexin binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂). 100 μl of this suspension (1 × 10⁶ cells) was removed to a new 1.5-ml Eppendorf tube. 6 μl of annexin-V-antibody was added to each tube and gently mixed. Tubes were incubated at room temperature for 15 min. After incubation, 400 μl of annexin binding buffer (containing 1 ng/ml propidium iodide (PI)) was added to each tube. Samples were gently mixed, placed on ice, and analyzed by flow cytometry within 60 min. Flow cytometry was performed on a BD Biosciences Canto with excitations of 488 and 633 nm. Emission wavelengths detected included >530 nm (YFP), 660 nm (allophycocyanin), and 670 nm (PI). Data analysis was performed using WinMDI 2.8 software program (written by Joseph Trotter, Scripps Research Institute, La Jolla, CA) and FCS express V3 (De Novo Software, Los Angeles, CA).

**Flow Cytometry-based Physical Light Scatter Apoptosis Assay (Nuclei)**

Apoptosis detection by physical light scatter was carried out as previously described in detail (34). Briefly, nuclei were collected after transient transfection by hypotonic lysis and sucrose gradient purification. The highly purified nuclei were resuspended in propidium iodide stain solution. After staining, nuclei were analyzed via flow cytometry to detect forward scatter, side scatter, YFP fluorescence (excitation 488 nm, emission 530 nm) and propidium iodide fluorescence (excitation 488 nm, emission >590 nm). Data analysis was performed using WinMDI 2.8 software program (written by Joseph Trotter, Scripps Research Institute) and FCS express V3 (De Novo Software, Los Angeles, CA).

**Immunoprecipitation**

PC12 cells grown in 75-cm² flasks were washed 3 times with PBS and solubilized in 400 μl of ice-cold immunoprecipitation buffer (1% Nonidet P-40 in 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, including the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), and leupeptin (10 μg/ml)) by gentle rocking for 1 h at 4 °C. Insoluble material was removed by centrifugation at 10,000 \( \times g \) for 10 min. All immunoprecipitation steps were performed at 4 °C.

**HA Pulldown**—HA pulldown was performed according to manufacturer’s instructions (Profound HA immunoprecipitation kit, Pierce). Briefly, protein extracts (800 μg) were incubated with agarose-coupled HA antibody rotating overnight at 4 °C. Immunoprecipitated proteins were eluted from the beads using elution buffer, gel loading buffer was added, and samples were heated at 100 °C for 5 min.

**Bcl-2-mediated Agarose A/G Pulldown**—Protein extracts (800 μg) were incubated with primary antibody Bcl-2 N19 (8 μl) with agarose A/G beads (50 μl). Samples were incubated overnight at 4 °C, and subsequently each immunoprecipitation was collected by centrifugation at 10,000 \( \times g \) for 1 min and
washed 3 times with ice-cold immunoprecipitation buffer and once with PBS (pH 7.4). For SDS-polyacrylamide gel electrophoresis of the immunoprecipitated proteins, agarose A/G beads were heated at 100 °C for 5 min in SDS-polyacrylamide gel electrophoresis buffer (each 100 ml of buffer contained 3 g of Tris, 8 g of SDS, 2.5g dithiothreitol, 0.05g bromphenol blue, 40% (v/v) glycerol).

BH4 Peptide Immunoprecipitation

PC12 cells were transiently transfected with HA-FKBP38 construct for 24 h as described above. Cells were lysed in non-denaturing lysis buffer, and total cell proteins were collected. Immunoprecipitation was performed using a HA antibody pulldown (Profound HA immunoprecipitation kit, Pierce), 800 μg of total protein, and 2 ng/μl BH4 or BH4 scramble peptide. After an overnight incubation rotating at 4 °C, proteins were eluted and utilized for polyacrylamide gel electrophoresis.

GelCode Stain

After immunoprecipitation and polyacrylamide gel electrophoresis, gels were stained using GelCode (Pierce) following the manufacturer’s instructions. Briefly, gels were washed 3 times with deionized water for 15 min. After washes, gels were incubated with ~50 ml of GelCode (for 8.3 × 7.3 cm gel) for 1 h. After staining, gels were rinsed three times with deionized water and then incubated overnight in water at room temperature. After an overnight wash, stained gels were placed on a light box, and digital images were taken using UVP 12bit monochrome digital Chemi HR camera (UVP, Upland, CA).

Statistical Analysis

Experiments were performed in triplicate (intra-assay variability). Final results were statistically evaluated on the average calculated of the three independent replicate experiments. Statistical differences between groups were assessed using Student’s t test. An α level below 5% (p < 0.05) between groups was considered statistically significant.

RESULTS

Apoptosis Induction after Transient Expression of Bcl-2 or Bcl-2ΔBH4—Rat PC12, human HeLa, mouse B104, and human HEK293 cells were transiently transfected with YFP, Bcl-2/YFP, or Bcl-2ΔBH4/YFP. After transient transfection, cells were stained with annexin-V- and propidium iodide and analyzed by flow cytometry. Cells expressing the transfected constructs, as determined by YFP fluorescence, were gated (R1), and the degree of apoptosis specifically in the R1 population was determined by assessing the number of cells that were simultaneously annexin-V-positive and propidium iodide-negative (Fig. 1A) (see supplemental Fig. 1 for an example of R1 gating). Analyses performed on three independent experiments revealed that all cell lines assayed underwent a significant degree of apoptosis upon transient transfection of Bcl-2/YFP or Bcl-2ΔBH4/YFP as compared with cells transfected with the YFP control vector. Western blot analysis detecting Bcl-2 in total protein extracts from non-transfected PC12, HeLa, B104, and HEK293 cells revealed different degrees of endogenous Bcl-2 expression in these cell lines, with the highest expression in HeLa cells and levels below the limit of detection in PC12 cells (Fig. 1B). Because induction of apoptosis by transient expression of exogenous Bcl-2 seemed not to be restricted to any specific cell line and was independent of endogenous Bcl-2 expression, we elected to focus our subsequent studies on PC12 cells. Next, we determined whether the fusion protein Bcl-2/YFP used in these experiments retained function similar to wild type Bcl-2. To test this, PC12 cells stably expressing either Bcl-2 or Bcl-2/YFP were exposed to serum withdrawal or staurosporine treatment. Both of these apoptosis-inducing stimuli have been shown to be attenuated by stable, mitochondrial compartment-localized Bcl-2 in PC12 cells (7, 8). Both stably expressed wild type Bcl-2 and Bcl-2/YFP provided significant protection from cell death after 24 h of serum withdrawal or 24 h of treatment with staurosporine (supplemental Fig. 2A). In addition, both stably expressed Bcl-2 and Bcl-2/YFP shared a non-nuclear subcellular localization (supplemental Fig. 2B).

Nuclear Localization of Bcl-2 or Bcl-2ΔBH4 Is Associated with a Significant Increase in Apoptosis—The representative Western blot shown in Fig. 2A compares the subcellular distribution of exogenously expressed Bcl-2 and Bcl-2ΔBH4 after either transient or stable transfection. Although transiently expressed Bcl-2 localized to both nuclear and non-nuclear fractions, transiently expressed Bcl-2ΔBH4 solely localized within
the nuclear fraction. Conversely, stably expressed Bcl-2 was predominately found in the non-nuclear protein fraction, whereas expression of Bcl-2ΔBH4 in the non-nuclear fraction was not detected after long-term growth in selection medium. Purity of nuclear and non-nuclear fractions was confirmed by re-probing with antibodies specific for nuclear and non-nuclear proteins (IκBα for cytosolic, Pan Lamin for nuclear, and calnexin for ER) (Fig. 2A). Although satisfactory separation of nuclear from non-nuclear fractions was confirmed, the small degree of calnexin identified in the nuclear fraction was expected since the ER is contiguous with the outer nuclear envelope (35) and our method of nuclei isolation preserves the outer nuclear membrane (34).

Fig. 2B shows representative confocal photomicrographs of nuclei isolated from PC12 cells transiently transfected with either Bcl-2/YFP or Bcl-2ΔBH4/YFP. These isolated nuclei showed a perinuclear association of Bcl-2 and Bcl-2ΔBH4, indicating that nuclear Bcl-2 is retained through the process of nuclei isolation. To investigate the extent of apoptosis in cells bearing Bcl-2 and Bcl-2ΔBH4 at the nuclear compartment, we employed a technique recently developed by us whereby flow cytometry-based analysis of physical light scattering properties (forward versus side scatter) is used to determine apoptotic status of isolated nuclei (34). Using this technique coupled with transfection of Bcl-2/YFP and Bcl-2ΔBH4/YFP fusion proteins, it was possible to compare apoptosis in both populations of nuclei that contained and lacked the presence of Bcl-2 or Bcl-2ΔBH4. Analysis from three independent experiments using the physical light scatter technique on nuclei isolated from Bcl-2/YFP and Bcl-2ΔBH4/YFP transiently transfected cells showed that there was a significant increase in apoptosis in nuclei bearing Bcl-2 (46 ± 1.73% as compared with 12 ± 3% in nuclei devoid of Bcl-2) (Fig. 2C). Similarly, there was a significant increase in apoptotic nuclei bearing Bcl-2ΔBH4 (50.7 ± 2.7% as compared with 13.74 ± 1.46% in nuclei devoid of Bcl-2ΔBH4) (Fig. 2D) (representative physical light scatter dot plots are shown in supplemental Fig. 3, A and B).

**Cell Death Induced by Transiently Transfected Bcl-2 Is Affected by Modulation of FKBP38 Expression Levels**—Cell fractionation (nuclear versus non-nuclear) was utilized in conjunction with Western blotting to determine changes in Bcl-2 and Bcl-2ΔBH4 localization after transient co-expression with FKBP38 (Fig. 3A). Transfection of Bcl-2 and FKBP38 reduced nuclear compartment-associated Bcl-2 and increased nuclear FKBP38. Co-transfection of Bcl-2ΔBH4 and FKBP38 increased nuclear compartment-associated FKBP38 but failed to decrease the level of nuclear Bcl-2ΔBH4. Flow cytometry-based counting of nuclei was also used to determine the percent of nuclei bearing Bcl-2 or Bcl-2ΔBH4 after transient transfection in the presence or absence of co-transfected FKBP38. As shown in Fig. 3B and consistent with the Western blot data shown in Fig. 3A, the addition of FKBP38 significantly reduced the percent of Bcl-2-positive nuclei from 26 ± 2.51% to 15 ± 2.05% but did not significantly alter the percent of Bcl-2ΔBH4-positive nuclei (34 ± 1.76% in the absence of FKBP38 versus 36 ± 3.2% in the presence of FKBP38). Furthermore, physical light scatter analysis revealed a significant reduction (p < 0.01) in apoptosis, 41.7 ± 1.74% for nuclei bearing Bcl-2 in the absence of co-expressed FKBP38 compared with 22.9 ± 3.26% after FKBP38 co-transfection (Fig. 3C). On the other hand, no significant reduction in apoptosis was observed for nuclei bearing Bcl-2ΔBH4 after FKBP38 co-expression; 49.3 ± 3.0% in the absence of co-expressed FKBP38 compared with 53.6 ± 3.4% after FKBP38 co-transfection (Fig. 3D) (representative physical light scatter dot plots are shown in supplemental Fig. 3, C and D).

Fig. 4 shows the results from a flow cytometry-based annexin-V assay in which cells were transiently transfected with Bcl-2ΔBH4 (nuclear localized Bcl-2) in the presence or absence of a co-transfected Bcl-2 mutant that is selectively targeted to the mitochondrion (Mito-Bcl-2) (subcellular localization of both Bcl-2 constructs has previously been characterized (11, 30)). Transiently transfected Bcl-2ΔBH4 induced a signifi-
Cant increase (p < 0.01) in apoptosis as compared with cells transfected with Mito-Bcl-2 (Fig. 4A). Furthermore, co-transfection of Mito-Bcl-2 failed to prevent apoptosis induced by Bcl-2ΔBH4 (Fig. 4B). The results from this experiment suggest that the FKBP38-mediated rescue of cells described in Fig. 3C was likely due to the reduced presence of nuclear Bcl-2 rather than increased mitochondrial delivery of Bcl-2 by co-transfected FKBP38.

To further characterize the apoptotic role of nuclear associated Bcl-2, we examined the changes in subcellular distribution of Bcl-2 and the extent of apoptosis after suppression of endogenous FKBP38 expression using siRNA. As shown in Fig. 5A, FKBP38 expression was almost completely abolished 72 h after the addition of FKBP38 siRNA. Subcellular fractionation and Western blots performed after 24 h of transient transfection with Bcl-2 in cells pretreated for 72 h with siRNA for FKBP38 (Fig. 5B, left panel) revealed a dramatic decrease in non-nuclear Bcl-2 paralleled by an appreciable increase of nuclear compartment-associated Bcl-2. Similar experiments performed using Bcl-2ΔBH4 instead of full-length Bcl-2 revealed no significant difference in levels of nuclear associated Bcl-2ΔBH4 between cells pretreated with the FKBP38 siRNA as compared with cell pretreated with a non-targeted siRNA (Fig. 5B, right panel). Apoptosis measured by annexin-V assay performed in similarly treated cells revealed a significant increase (p < 0.05) in apoptosis in cells transfected with Bcl-2 but not Bcl-2ΔBH4 after FKBP38 siRNA knockdown (Fig. 5C).
FKBP38 Binds Bcl-2 Utilizing the Bcl-2 BH4 Domain—Because the addition of FKBP38 failed to remove Bcl-2ΔBH4 from the nuclear compartment (Fig. 3, A and B), we asked whether the BH4 domain of Bcl-2 was critical for binding to FKBP38. To address this question, we performed immunoprecipitations 48 h after transient co-expression of Bcl-2 and FKBP38 or Bcl-2ΔBH4 and FKBP38. Western blots performed on input (total non-denatured protein extract), IP (eluate from agarose beads with conjugated HA antibody), and beads (eluate from agarose beads without antibody) were probed for FKBP38, Bcl-2, and Bcl-2ΔBH4. Although Bcl-2 co-immunoprecipitated with FKBP38, Bcl-2ΔBH4 failed to co-immunoprecipitate (Fig. 6A).

This result was confirmed by performing a reciprocal co-IP using an anti-Bcl-2 antibody for pulldown (Fig. 6B). Lack of co-IP between FKBP38 and Bcl-2ΔBH4 suggested that the BH4 domain of Bcl-2 was involved in FKBP38 binding.
but did not distinguish whether the BH4 domain directly bound FKBP38 or simply stabilized the binding. Therefore, to determine whether the association between Bcl-2 and FKBP38 was directly mediated by the Bcl-2 BH4 domain, we synthesized a BH4 domain peptide and control BH4 domain scrambled amino acid sequence peptide (BH4scr) for use in FKBP38 immunoprecipitation assays. Incubation of the BH4 domain peptide with total cell extracts from PC12 cells that were previously transiently transfected with FKBP38 revealed that the BH4 domain peptide co-immunoprecipitated with FKBP38 (Fig. 6C). In addition, immunoprecipitation performed using the control BH4scr peptide failed to co-immunoprecipitate with FKBP38 (Fig. 6D). Because of the lack of an antibody that detects BH4scr peptide, we performed gel staining using GelCode stain reagent for detection of loaded (Input) and bound (IP) proteins. As shown in Fig. 6C, GelCode stain revealed the BH4 peptide in input and IP lanes, whereas the BH4scr peptide was detected solely in the input but not in the IP fraction (Fig. 6D).

DISCUSSION

We previously reported evidence of a pro-apoptotic role for nuclear compartment-associated Bcl-2. Previous studies have demonstrated that Bcl-2 localizes to multiple organelles including the mitochondria, ER, and nuclear envelope (13). Although most studies have focused on the anti-apoptotic role of mitochondrial localized Bcl-2, recent evidence has suggested that Bcl-2 can act in a pro-apoptotic fashion (29–31). Our results indicate that one putative subcellular localization where the pro-apoptotic action of Bcl-2 can be exerted is at the nuclear compartment, specifically the perinuclear area. These studies have far-reaching implications because they reveal a dual role for Bcl-2: one, when localized at the nuclear compartment, Bcl-2 is incompatible with long-term cell survival due to induction of apoptosis, and two, the Bcl-2 BH4 domain plays a crucial role in Bcl-2 intracellular transport.

To address the question as to whether Bcl-2 has a proapoptotic role after nuclear association, we applied a flow cytometry-based assay recently developed by us (34) for characterization of apoptosis in isolated nuclei. This assay allows detection of nuclear compartment-associated proteins while simultaneously determining the apoptotic nature of the cell from which the nucleus was isolated. Analysis of nuclei isolated from PC12 cells transiently transfected with Bcl-2 or Bcl-2ΔBH4 (Fig. 2B) revealed a significant increase in the percent of apoptotic nuclei specifically in those nuclei bearing Bcl-2 or Bcl-2ΔBH4 as compared with nuclei devoid of Bcl-2 or Bcl-2ΔBH4 (Fig. 2, C and D). Although these findings linked the presence of nuclear associated Bcl-2 and Bcl-2ΔBH4 to the induction of apoptosis, they did not provide conclusive evidence for a causal relationship between the two events. However, both Western blot and flow cytometry-based analysis of isolated nuclei showed that the presence of FKBP38 reduced the presence of nuclear Bcl-2 (Fig. 3, A and B) while significantly reducing apoptosis (Fig. 3C). On the other hand, co-expressed FKBP38 failed to remove nuclear compartment-associated Bcl-2ΔBH4 (Fig. 3, A and B) and failed to enhance the survival of cells expressing Bcl-2ΔBH4 (Fig. 3D). Collectively these results suggest that there is either a causal link between the presence of Bcl-2 at the nuclear compartment and initiation of apoptosis in PC12 cells or that the added FKBP38 increased mitochondrial-localized Bcl-2 and the increased survival observed was due to protection exerted by the increased mitochondrial Bcl-2.

To directly test whether the decrease in apoptosis observed after the addition of FKBP38 was due to increased mitochondrial Bcl-2, we performed co-transfections with Bcl-2ΔBH4 and a mitochondrial-targeted Bcl-2 (Mito-Bcl-2). Our result show that directly adding Bcl-2 to the mitochondria in the presence of nuclear trapped Bcl-2 (Bcl-2ΔBH4) failed to significantly protect cells from nuclear Bcl-2-mediated apoptosis (Fig. 4B). These data indicate that the increase in cell survival observed via co-expression of FKBP38 and Bcl-2 but not FKBP38 and Bcl-2ΔBH4 was predominantly mediated by reduced pro-apoptotic nuclear Bcl-2 rather than increased anti-apoptotic mitochondrial Bcl-2. A similar conclusion could be drawn from the data shown in Fig. 1 where transient expression of Bcl-2 induces apoptosis in cell lines that express substantial levels of endogenous Bcl-2, thus suggesting that the presence of endogenous Bcl-2 (mostly mitochondrial) does not protect cells from apoptosis induced by transiently expressed Bcl-2 (which localizes substantially at the nucleus). Furthermore, suppression of endogenous FKBP38 expression by
siRNA (Fig. 5) shifted the localization of Bcl-2 almost completely to the nuclear compartment and made transiently transfected Bcl-2 as toxic as Bcl-2ΔBH4, which is solely nuclear. These results provide evidence for a causal relationship between the toxicity of transiently transfected Bcl-2 and its presence at the nuclear compartment. Collectively, these results also suggest that mitochondrial Bcl-2 is unable to block apoptosis triggered by nuclear Bcl-2. Although this observation argues for a novel mechanism of initiation of apoptosis triggered by nuclear Bcl-2 that bypasses the mitochondrial checkpoint, further studies are needed to elucidate the exact molecular pathways involved.

In characterizing the pro-apoptotic nature of nuclear Bcl-2, we generated evidence that the BH4 domain of Bcl-2 was critically involved in regulating Bcl-2 movement, likely through facilitating interaction with the chaperone protein FKBP38. To determine whether the Bcl-2 BH4 domain was the critical domain responsible for Bcl-2-mediated binding to FKBP38, we performed co-immunoprecipitation experiments with FKBP38 and Bcl-2, Bcl-2ΔBH4, and a synthetic BH4 domain peptide (Fig. 6, A–D). Although our results confirmed previously published reports that Bcl-2 and FKBP38 co-immunoprecipitate (Fig. 6, A and B), they also showed for the first time that removal of the Bcl-2 BH4 domain results in loss of co-immunoprecipitation with FKBP38 (Fig. 6, A and B). This finding in conjunction with the observation that FKBP38 fails to remove Bcl-2ΔBH4 from the nuclear compartment (Fig. 3, A and B) strongly suggests that the BH4 domain of Bcl-2 is critically involved in regulating the binding between Bcl-2 and FKBP38 and the subsequent delivery of Bcl-2 to the mitochondrion. Indeed, we found that a BH4 domain peptide co-immunoprecipitated with FKBP38, thus providing direct evidence that the BH4 sequence of Bcl-2 is sufficient to mediate binding to FKBP38. This is not surprising given the extensive literature describing the BH4 domain as a “docking” domain that allows Bcl-2 to interact with several heterologous proteins (36, 37).

In light of these data it is interesting that another group using a prokaryotic overexpression approach found that the Bcl-2 loop domain, located next to the BH4 domain, was directly responsible for mediating FKBP38/Bcl-2 binding (19). Although this report appears to contradict our current findings, both results could be explained by the fact that the loop domain is a flexible structure that positively displaces the Bcl-2 BH4 domain for heterologous protein interactions (38–40). It is, therefore, possible that deletions within this domain induced conformational changes that blocked or hindered access of FKBP38 to the Bcl-2 BH4 domain. This argument is strengthened by the fact that the same authors failed to identify an exact binding domain sequence within the loop domain and found that several deletions of non-congruent sections of the loop domain resulted in alteration in FKBP38 binding. Therefore, the previous finding that FKBP38 binds to the loop domain of Bcl-2 is not a direct contradiction with our current results which indicate that the BH4 domain of Bcl-2 is the critical domain required for Bcl-2/FKBP38 binding. Nonetheless, the mechanism through which both domains are critically involved in stabilization and maintenance of Bcl-2/FKBP38 binding remains to be established.

In conclusion, we have shown that nuclear compartment-associated Bcl-2 promotes rather than protects cells from apoptosis. As schematically illustrated in Fig. 7, the ability to regulate Bcl-2 movement and, thus, dictate an anti-apoptotic (mitochondria) or pro-apoptotic (nuclear) mechanism appears to depend upon proper interaction with the chaperone protein FKBP38, an interaction in which the Bcl-2 BH4 domain plays a critical role. This observation offers a significant target for future therapies aimed at eliminating Bcl-2-expressing cells or enhancing Bcl-2-mediated cell survival. The future development of possible BH4 domain mimetic molecules could be proposed to disrupt Bcl-2/FKBP38 interaction and, thus, selectively target Bcl-2 to the nucleus to induce apoptosis, specifically in high Bcl-2-expressing cells such as several types of human cancers (41). On the other hand, neurons and supporting glia expressing high levels of Bcl-2 after transient ischemic attack/stroke (42, 43) could potentially be saved by promoting mitochondrial rather than nuclear association of Bcl-2. Feasibility of pharmacologically relevant techniques that will allow such manipulation of Bcl-2 localization remains to be determined.

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