Inhibition of Transcription Factor Activity by Nuclear Compartment-associated Bcl-2

Cynthia A. Massaad, Bryce P. Portier, and Giulio Taglialatela

From the Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-1043

Using a reporter gene assay in PC12, HEK293, HeLa, and NIH-3T3 cells, we show that the anti-apoptotic protein Bcl-2 significantly inhibits transcriptional activation of various transcription factors, including NFκB, AP1, CRE, and NFAT. A Bcl-2 mutant lacking its BH4 domain (∆BH4) also inhibited transcription, whereas a Bcl-2 mutant lacking its transmembrane domain (∆TM) was ineffective. Furthermore, Bcl-2 chimeric proteins containing transmembrane domains from the mitochondrial protein monoamine oxidase B (MaoB) or the endoplasmic reticulum protein cytochrome b$_5$ showed no effect on transcription factor activity. Subcellular localization studies showed that under conditions of transient transfection, the active Bcl-2 forms (wild type and ∆BH4) were predominantly found in the nuclear fraction, whereas the non-active forms (∆TM, MaoB, and cytochrome b$_5$) were in the non-nuclear fraction. Additionally, stably expressed Bcl-2 loses its ability to inhibit transcriptional activation and localizes predominantly to the non-nuclear fraction. Expression of FKBP38 (a chaperone that shuttles Bcl-2 to the mitochondria) removes co-expressed Bcl-2 from the nuclear fraction and reverses its effect on transcription factor activity. Finally, using an inducible gene expression system, we show that nuclear compartment-associated Bcl-2 prevents entry of NFκB subunits to the nucleus without affecting NFκB release from its cytosolic inhibitory subunit IκBα. These results suggest that (a) Bcl-2 suppresses transcriptional activity of multiple transcription factors; (b) Bcl-2 does not interfere with NFκB activation but prevents entrance of its active subunits to the nucleus; (c) membrane anchoring is required for this function of Bcl-2; and (d) association of Bcl-2 with the nuclear compartment is also necessary. We speculate that nuclear compartment-associated Bcl-2 may affect nuclear trafficking of multiple factors necessary for transcriptional activity.

Apoptosis is an evolutionarily conserved physiological process of cell death by which any superfluous or potentially dangerous cell may be eliminated (1, 2). Prevention, initiation, and execution of apoptosis is a complex event, involving the coordinated action of many effector proteins (3). The best characterized class of proteins regulating apoptosis is the Bcl-2 protein family (4, 5). Proteins belonging to this family are characterized by the presence of one or more homologous domains named Bcl-2 homology domains 1–4 (BH1–4) (6). Among these, the BH4 domain is responsible for interaction with non-Bcl-2 proteins such as Ras, Raf-1, and calcineurin (7), and the transmembrane domain (TM) is responsible for anchoring Bcl-2 to intracellular membranes, including mitochondria, endoplasmic reticulum (ER), and the nuclear membrane (8–10).

The Bcl-2 family of proteins can be divided into three major groups based on their role in modulating apoptosis: the anti-apoptotic members including Bcl-2 and Bcl-XL, the proapoptotic members including Bax and Bak, and the BH3-only members including Bim and Bad (5). Although the mechanism of action of Bcl-2 is not completely understood, it is believed to involve at least in part inhibition of cytochrome c release from the mitochondria (11, 12), regulation of free radical production (13–15), and modulation of calcium homeostasis (16, 17). Furthermore, Bcl-2 has been associated with calcineurin, nuclear factor κB (NFκB), and p53, possibly modulating their function (18–20).

We have previously shown that during aging and oxidative stress, Bcl-2 is overexpressed and found localized predominantly at the nuclear membrane (21, 22). We further showed that nuclear Bcl-2 failed to protect cells from apoptosis induced by oxidative stress and hypothesized that the function of nuclear Bcl-2 may not be anti-apoptotic, at least in the aged or oxidatively stressed central nervous system (22). However, the question as to the role of Bcl-2 within the nuclear compartment remains unanswered.

Although Bcl-2 has been reported in both the ER and nuclear membranes, more emphasis has been put on its role as a mitochondrial protein. More recently, growing evidence is emerging as to the role of ER-associated Bcl-2 in anti-apoptosis. A mutant Bcl-2 targeted solely to the ER membrane has been shown to be fully functional as an anti-apoptotic protein, inhibiting caspase activation and sequestering Bad to prevent it from activating the pro-apoptotic Bax (23). Currently, research involving nuclear Bcl-2 is limited to localization studies but lacks analysis of nuclear Bcl-2 function (8–10). Recently, work by Shirane and Nakayama (24) showed that FKBP38, an inherent calcineurin inhibitor containing a mitochondrial targeting sequence, co-immunoprecipitates with Bcl-2 and is required for proper localization of Bcl-2 to the mitochondrion and Bcl-2 anti-apoptosis function. Notably, these authors showed that mutation or elimination of FKBP38 results in cell death because of mislocalization of Bcl-2, mostly to the nuclear membrane. This unexpected observation raised a very important

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‡ To whom correspondence should be addressed: Dept. of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555-1043. Tel.: 409-772-1679; Fax: 409-772-1861; E-mail: gtaglia@utmb.edu.

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In this study we report that high levels of Bcl-2 lead to accumulation of Bcl-2 at the nuclear membrane and result in a decrease in transcription factor activity including NFκB, AP1, CRE, and NFAT. We also show that the transactivation of a minimal promoter from SV40 thymidine kinase or a full-length mouse promotor is affected by high expression of Bcl-2, suggesting a global effect of nuclear Bcl-2 on transcription. Furthermore, we show that whereas mitochondrial- or ER-targeted Bcl-2 mutants fail to produce any effect on transcription factors, co-expression of FKBP38 with wild type Bcl-2 removes Bcl-2 from the nucleus and abolishes the transcriptional effects of Bcl-2, suggesting that Bcl-2 nuclear localization and its effect on nuclear transcription factor activity may be associated. Finally, we show that Bcl-2 prevents the translocation of active NFκB subunits to the nuclear compartment without affecting the process of NFκB activation, suggesting a role of nuclear compartment-associated Bcl-2 on nuclear trafficking. These results are important to define the role of nuclear Bcl-2 in the aged and oxidatively stressed central nervous system, where cell death and significant alterations of transcription factor activity may have been reported (25–29).

EXPERIMENTAL PROCEDURES

DNA Constructs

pMKItNeo-Bcl-2-ΔBH4 was generated by introducing a start codon at amino acid position 25 of parental Bcl-2, flanked by an XhoI site. The obtained fragment was then digested with XhoI/EcoRI and re-cloned into pMKIt-Neo. pMKItNeo-Bcl-2-3TM was generated by introducing a stop codon at amino acid position 223 of parental Bcl-2, flanked by an XhoI site. The obtained fragment was then digested with XhoI/NotI and re-cloned into pMKIt-Neo/Bcl-2 was a kind gift of Dr. S. May, Bcl-2-cytochrome b, and Bcl-2-MaeB was a kind gift of Dr. C. W. Distelhorst, FKBP38 was a kind gift of Dr. K. I. Nakayama. All SEAP vectors were purchased from Clontech.

Cell Culture

Rat pheochromocytoma cells (PC12), human embryonic kidney 293 cells (HEK 293), mouse fibroblast cells (3T3), and human epithelial cells (HeLa) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in a humidified atmosphere with 5% CO2 in RPMI 1640 supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin (Cellgro, Herndon, VA) (PC12) or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (HEK293-HeLa-3T3).

Transfections

All transfections were performed using a liposomal mediated plasmid introduction. The liposome used was DMRIE-C (Invitrogen, Carlsbad, CA). Cells at 40 to 50% confluence received a total of 1.2 pmol/ml of DNA coupled to DMRIE-C at a ratio of 1:3 and diluted in Opti-MEM (Invitrogen). 3 h post-transfection, the liposome/DNA mixture was replaced with fresh culture medium and the cells were allowed at least 24 h of recovery. Stable transfectants were generated using the same transfection technique, followed by the addition of the proper selective antibiotic to the culture medium (Geneticin, Invitrogen).

Reporter Assay

The secreted alkaline phosphatase (SEAP) assay (Clontech) was used as a measure of transcription factor activity. Plasmids containing the SEAP gene only or the SEAP gene downstream of enhancer sequences specific to binding of NFκB, AP1, CRE, or NFAT were cotransfected with Bcl-2, Bcl-2-ΔBH, or Bcl-2-3TM. 48 h later, SEAP activity was assayed directly from the culture medium using the Great Escape Chemiluminescent Detection Kit according to manufacturer’s instructions (Clontech). SEAP levels are a direct measurement of the effect of Bcl-2 and Bcl-2 mutants on the enhancer activity.

Cell Extracts and Subcellular Fractionation

For gene expression verification, cells were lysed in SDS lysis buffer containing 5 mM EDTA, 50 mM Tris, 2% SDS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% protease mixture inhibitors (Sigma). Following lysis, cells were sonicated for 15 s, and then centrifuged at 20,000 × g for 5 min. For protein localization experiments, nuclear and non-nuclear fractions were prepared using the Biovision Inc. Cytosolic/Nuclear fractionation kit according to manufacturer’s suggested protocol (Mountain View, CA). Briefly, cells were suspended in hypotonic buffer and lysed with the proprietary detergent from the kit. Samples were spun at 800 × g for 10 min at 4 °C. The supernatant was collected, spun 10 min at 1000 × g to remove any remaining nuclei, and then transferred to a new tube (non-nuclear protein fraction). The original pellet was resuspended in Sigma’s Nuclei pure prep lysis buffer (Sigma) and nuclei isolation was carried out following the manufacturer’s recommended protocol. Briefly, following a 10-min incubation in lysis buffer the nuclei were layered on a 1.8 M sucrose gradient and spun at 15,500 rpm for 25 min at 4 °C. The nuclear pellets were re-suspended in cold phosphate-buffered saline and spun at 20,800 × g for 2 min at 4 °C, resuspended in SDS lysis buffer (5 mM EDTA, 50 mM Tris, 2% SDS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1% protease mixture inhibitors), and sonicated 15 s (nuclear proteins).

Immunoblotting

Protein samples containing 30 µg of proteins as determined by the BCA assay (Pierce, Rockford, IL), were subjected to SDS-PAGE using 12% gels, followed by electrophoretic transfer to nitrocellulose-backed membranes (Bio-Rad). Membranes were then incubated with 1:1000 (v:v) anti-Bcl-2 polyclonal antibody (BD Pharmingen, San Diego, CA), 1:10,000 anti-FKBP38 polyclonal antibody (kind gift of Dr. K. I. Nakayama), 1:50,000 anti-poker monoclonal Antibody (Sigma), 1:2000 PanLamin (kind gift of Dr. A. Fields), or 1:2000 Igs (Santa Cruz Biotechnology, Santa Cruz, CA). This initial incubation was then followed by a horseradish peroxydase-conjugated secondary antibody (Bio-Rad) against rabbit IgG (for polyclonal primaries) or mouse IgG (for monoclonal primaries). Detection was achieved using ECL (Amersham Biosciences).

Confocal Microscopy

Cells were grown on glass coverslips in 12-well plates. Following transient transfection as described above, cells were fixed with 4% paraformaldehyde or 0.5% glutaraldehyde or formaldehyde or was fixed live in normal growth media without phenol red. Organelle markers used for imaging mitochondria, ER, and nucleus include MitoTracker Deep Red, ER-Tracker, and 4,6-diamidino-2-phenylindole, respectively (Molecular Probes Inc.). Images were obtained using a Zeiss LSM510 META advanced laser scanning confocal microscope with laser excitations at 633 (MitoTracker), 351 (ER Tracker and 4,6-diamidino-2-phenylindole), 488 nm (GFP), and long pass filters >650 nm (MitoTracker), between 385 and 545 nm (ER Tracker and 4,6-diamidino-2-phenylindole) and >505 nm (GFP). All imaging analysis was performed using Meta-morph imaging software version 6.0 (Universal Imaging Corp. Downingtown, PA).

GeneSwitch™ Inducible Expression System

DNA Constructs—The GeneSwitch™ system (Invitrogen) is a gene regulation system that controls gene expression through treatment with a synthetic hormone. This system consists of two main genes: one encoding the GeneSwitch™ regulator protein driven by a constitutive promoter and another encoding for the inducible gene of interest. pSwitch (the plasmid vector encoding the GeneSwitch™ regulator protein) and pGene/V5-His/mbcl-2a (an inducible plasmid vector containing our gene of interest) was produced as follows: 5’ EcoRI and 3’ NotI were inserted into the Bcl-2 gene by PCR from its original vector, pMKITNeo/mbcl-2a. The PCR product was then digested with EcoRI and NotI and ligated into the EcoRI/NotI site of the inducible plasmid vector pGene/V5-His/mbcl-2a, the inducible plasmid vector containing our gene of interest. This construct was transfected into the HeLa cell line, which expresses the inducible protein. Stable transfectants were then selected using 500 µg/ml Zeocin, and Zeocin-resistant colonies were screened for successful transfection. All other conditions were the same as for stable transfectants (non-zeocin).
Nuclear Bcl-2 Inhibits Transcription Factor Activity

RESULTS

Bcl-2 Inhibits NFκB Transcriptional Activity—We have previously shown that Bcl-2, when overexpressed by transient transfection in PC12 cells, significantly decreases NFκB activity, as measured by the SEAP reporter gene system (30). We have also shown that this phenomenon is independent of Bcl-2 S70 phosphorylation, suggesting that the effect of Bcl-2 on NFκB transcription has different phosphorylation requirements than its anti-apoptotic activity (30). Here we confirmed and extended that original observation as reported in Fig. 1A, which shows a dose-response curve illustrating that the Bcl-2-promoted decrease of NFκB activity as measured by the SEAP reporter gene assay is proportional to the amount of transfected/expressed Bcl-2 (Fig. 1B). Based on these results, 0.6 pmol of Bcl-2 expression vector was used in all further transfection experiments. Fig. 1C shows a time course experiment where NFκB activity in PC12 cells was determined by the SEAP reporter gene assay as a function of time after transient Bcl-2 transfection. A significant reduction of NFκB transcriptional activity was observed starting 24 h after Bcl-2 transfection and further increased 48 h after Bcl-2 transfection. This time course was consistent with the appearance of detectable levels of exogenously expressed Bcl-2 after transient transfection (Fig. 1D). Based on these results, 48 h after Bcl-2 transfection was selected as an end point in all further experiments.

Inhibition of NFκB Activity by Bcl-2 Depends on the Integrity of the Transmembrane Domain of Bcl-2—To identify Bcl-2 domains required for affecting NFκB transcriptional activity, we used Bcl-2 deletion mutants lacking either the BH4 domain (amino acids 1–24, Bcl-2-ΔBH4) or the transmembrane domain TM (amino acids 223–247, Bcl-2-ΔTM). The BH4 domain is responsible for Bcl-2 interactions with heterologous proteins (6, 31–33) and could thus mediate direct interaction with NFκB and the TM domain is necessary for anchoring Bcl-2 to intracellular membranes, including mitochondrial, ER, or nuclear membranes (6, 34, 35) (Fig. 2A). Fig. 2B shows the effect of these mutants on NFκB activity as determined by the SEAP reporter gene assay. Bcl-2-ΔBH4 effectively inhibited NFκB activity, indicating that the BH4 domain is not involved. On the other hand, Bcl-2-ΔTM failed to decrease NFκB activity, indicating the necessity for Bcl-2 to be anchored to a membrane to affect NFκB transcription. As both the BH4 and TM domains have been shown to be necessary for mediating the anti-apoptotic activity of Bcl-2, their different requirement in the Bcl-2-mediated decrease of NFκB transcriptional activity further suggests that this effect of Bcl-2 is acted in a fashion independent of its classical role as an anti-apoptotic protein (30). The representative Western blot in Fig. 2C shows the typical expression of the Bcl-2 mutants used in these experiments 48 h after transient transfection. Bcl-2-ΔTM expression was consistently higher than Bcl-2-ΔBH4 or wild type Bcl-2. However, because Bcl-2-ΔTM failed to show any effect on NFκB-mediated transcription, these results would rule out the possibility that the differential effect of the two Bcl-2 mutants may be because of different degrees of expression following transient transfection.

This concept is further illustrated in the dose-response curves shown in Fig. 2D. Bcl-2-ΔTM did not inhibit transcription factor activity at any of the doses of transfected DNA used in these experiments, whereas Bcl-2-ΔBH4 decreases NFκB-promoted transactivation at all concentrations in a dose-dependent manner.
Bcl-2 Inhibits Multiple Transcription Factors and Promoter Activities—

To investigate whether the reported transcriptional effect of Bcl-2 was limited to NF<sub>H9260</sub>B, a similar experimental paradigm as above was applied to cells transiently co-transfected with a SEAP reporter gene vector enhanced by AP1, CRE, or NFAT. An additional SEAP construct devoid of any enhancer element but still retaining a minimal promoter from SV40 thymidine kinase (pTAL-SEAP) was also employed. Fig. 3 shows the results from these experiments. All transcription factors and promoters used were similarly affected by the co-transfected Bcl-2 and Bcl-2 mutant expression vectors: wild type Bcl-2 and Bcl-2<sup>-/H9004</sup> BH4 decreased SEAP release into the culture medium, whereas Bcl-2<sup>-/H9004</sup> TM had no effect, regardless of the enhancer present upstream of the TAL promoter. The Western blots shown in Fig. 3B confirmed that all forms of Bcl-2 were expressed at comparable levels, thus again ruling out the possibility that the diverse effects of the different Bcl-2 mutants may be because of varying levels of expression. Furthermore, Fig. 3C shows that transient co-expression of Bcl-2, Bcl-2<sup>-/H9004</sup> BH4, or Bcl-2<sup>-/H9004</sup> TM with a luciferase reporter gene under the transcriptional regulation of a rat full-length promoter (angiotensin II) returned similar results in terms of transcriptional effects elicited by Bcl-2 and Bcl-2 mutants. These results suggest that the transcriptional effect exerted by Bcl-2 is not limited to NFκB activity; rather, it extends to other transcription factors and minimal (pTAL) or full-length promoters. In addition, the angiotensin II promoter/luciferase construct was also affected, further indicating that the reported transcriptional effect of Bcl-2 is not limited to the SEAP reporter gene constructs (all of which share the pTAL minimal promoter), nor to the SEAP gene itself. Furthermore, to rule out the possibility of an effect limited to PC12 cells, all experiments were repeated using NIH-3T3, HEK 293, and HeLa cells (Fig. 3D and E), yielding similar results.

Restricting Bcl-2 Expression to the Mitochondrion or ER Abolishes the Effects on Transcription on Bcl-2—

Because deletion of the TM domain negates the reduction induced by transiently transfected Bcl-2 on transcription factor activity, we reasoned that anchoring of Bcl-2 to cellular membranes is necessary for allowing this effect of Bcl-2. To further characterize this phenomenon, we investigated whether any of the known subcellular localizations of Bcl-2 (mitochondrion, ER, or nuclear membranes) were primarily involved. For this purpose, we used two Bcl-2 mutants: Bcl-2-MaoB, specifically targeted to the mitochondrial membrane, and Bcl-2-cytochrome b<sub>5</sub>, specifically targeted to the ER membrane (36) (Fig. 4A). When we co-transfected the NFκB-SEAP reporter construct with Bcl-2-MaoB or Bcl-2-cytochrome b<sub>5</sub> (Fig. 4B) we observed no effect on NFκB activity as compared with wild type Bcl-2, thus ruling out the possibility that these effects were because of anchoring of Bcl-2 to nuclear membranes.
out both the mitochondria and ER as sites of action for effects on transcriptional activity of the Bcl-2. This result suggested that Bcl-2 localized at the nuclear membrane may be primarily involved in decreasing transcription. Currently, there is no Bcl-2 mutant specifically targeted to the nuclear membrane available and therefore we could not test this conclusion directly. However, this idea is further supported by the results shown in Fig. 4C, where we determined by Western blot the subcellular distribution of wild type Bcl-2, Bcl-2-ΔBH4, and Bcl-2-ΔTM on the angiotensin full-length promoter activity, as measured by a luciferase reporter assay. Values are expressed as percent of control (vector-alone) and shown as mean ± S.D. of three replicates per group. B, NFκB transcriptional activity in HeLa, HEK 293, and NIH-3T3 cells as measured by the SEAP reporter gene system. Values are expressed as percent of control (vector-alone) and shown as mean ± S.D. of three replicates per group. E, Western blots confirming the expression of all Bcl-2 types in various cell lines. *, p < 0.01; **, p < 0.005; ***, p < 0.001; two-tailed unpaired Student’s t test. RLU, relative light units.

FKBP38 Reverses the Effect on Transcription Factor Activity by Bcl-2—To further confirm the involvement of nuclear Bcl-2 in decreasing transcription factor activity, we tested the effect of FKBP38 on the Bcl-2-promoted decrease in NFκB activity. The rationale behind this experiment is based on a recent report describing FKBP38, an inherent calcineurin inhibitor belonging to the FK506-binding protein family, as a chaperone responsible for proper localization of Bcl-2 to the mitochondria and ER. In the absence of FKBP38, Bcl-2 is aggregated around the nucleus and is unable to protect from cell death (24). In our experiments, FKBP38 was co-transfected with NFκB-SEAP and wild type Bcl-2 so as to determine its effect on the Bcl-2-promoted decrease in NFκB-driven transcription. Fig. 5A illustrates the results relative to these experiments showing that co-expression of FKBP38 significantly reverses the negative effect of wild type Bcl-2 on NFκB activity as determined by the SEAP reporter gene assay. The corresponding Western blot performed on total protein cell extracts shows expression of both transfected FKBP38 and Bcl-2 (Fig. 5B). Furthermore, detection of Bcl-2 by Western blot in nuclear protein extracts from PC12 transiently co-transfected with FKBP38 and Bcl-2 revealed a significant decrease of nuclear Bcl-2 at 48 h after transfection (which parallels the abolishment of the effect of Bcl-2 on NFκB activity as shown in Fig. 5A) followed by a
Bcl-2 Inhibits Nuclear Translocation of NFκB Subunits p50 and p65—PC12-Switch-mBcl-2α cells showed a dose-response increase of Bcl-2 expression when treated with increasing doses of the inducing hormone mifepristone (MFP). This result is illustrated in the Western blot shown in Fig. 6A, and indicates that the GeneSwitch™ system is background-free (first lane, non-induced sample) and properly inducible in PC12 cells. Fig. 6B shows that when Bcl-2 expression is induced in PC12-Switch-mBcl-2α cells and further co-transfected with an NFκB-SEAP reporter gene, NFκB-promoted transactivation is decreased, indicating that GeneSwitch™-promoted induction of Bcl-2 leads to similar effects as transiently transfected Bcl-2. Subcellular fractionation and Western blots from these cells (Fig. 6C) reveals a distribution of induced Bcl-2 in both nuclear and non-nuclear compartments. These blots also show decreased p50 and p65 levels in the nucleus of induced cells, indicating that NFκB activation (release from IkBα) is affected or transport of the active transcription factor to the nucleus is impaired. However, probing for IkBα in the cytosol of induced cells shows a decreased level of this inhibiting subunit, indicating that IkBα is being degraded and hence NFκB is being activated (37–39).

**FIG. 4.** A, confocal images from PC12 cells transiently transfected with GFP-Bcl-2-MaoB (targeted to the mitochondria) or GFP-Bcl-2-cytochrome b₅ (targeted to the ER), then loaded with either MitoTracker Red (mitochondrial marker) or ER tracker (ER marker). Controls include GFP-MaoB (vector with mitochondrial target, lacking Bcl-2) and GFP-cytochrome b₅ (vector with ER target lacking Bcl-2). r² = 1 as perfect correlation. B, NFκB activity detected by pNFκB-SEAP reporter gene assay in PC12 cells transiently transfected with Bcl-2-MaoB or Bcl-2-cytochrome b₅. Values are expressed as percent of control (Vector alone) and shown as mean ± S.D. of three replicates per group. Results are representative of at least three independent experiments. C, Western blot detecting Bcl-2 in nuclear and non-nuclear fractions of PC12 cells from the same experimental groups shown in Fig. 2B. Control subcellular fractionation include detection of IkBα (cytosolic protein) and PanLamin (nuclear protein). Control for equal loading was achieved by detection of β-actin. D, NFκB activity was detected by pNFκB-SEAP reporter gene assay in PC12 cells stably transfected with wild type Bcl-2. Values are expressed as percent of control (Vector alone) and shown as mean ± S.D. of three replicates per group. Results are representative of at least three independent experiments. E, Western blot detecting Bcl-2 in nuclear and non-nuclear fractions of PC12 cells from the same experimental groups shown in D. *** p ≤ 0.001; two-tailed unpaired Student’s t test.
DISCUSSION

Our results indicate that overexpressing Bcl-2 in PC12 cells leads to a significant decrease in NFκB-, AP1-, CRE-, or NFAT-promoted transcriptional activity. The effect of Bcl-2 could be observed even when the BH4 domain of Bcl-2 was deleted. Given that the BH4 domain has been reported to mediate interactions of Bcl-2 with heterologous proteins (6) and that its deletion does not affect the Bcl-2-promoted transcriptional inhibition, we speculate that Bcl-2 is unlikely to act by physically binding to the aforementioned transcription factors. Conversely, deletion of the TM domain of Bcl-2 completely abolished its effect on transcription. This would suggest that Bcl-2 requires insertion into cell membranes to affect transcription.

Classically, Bcl-2 has been described as being associated with membranes of mitochondria, ER, and nuclear envelope (8–10). Localization of Bcl-2 at either or all of these intracellular membranes could be mediating these effects, which prompted us to further investigate this phenomenon. Targeting Bcl-2 specifically to the mitochondria or the ER, using the TM domains of monoamine oxidase B and cytochrome b₅, respectively (36), abolished the effect of Bcl-2, indicating that restricting Bcl-2 expression to either the mitochondrion or the ER
renders Bcl-2 unable to affect transcription factor activity. In the absence of a sequence that would specifically target Bcl-2 to the nuclear membrane, we could not directly test the involvement of Bcl-2 nuclear localization. However, Western blots performed on subcellular protein fractions showed that a significant fraction of those Bcl-2 species that in our experimental model significantly reduced transcription factor activity (wild type and ΔBH4) was localized in the nuclear fraction, whereas the ineffective Bcl-2 mutant (ΔATM) was primarily in the non-nuclear fraction. Furthermore, stable expression of Bcl-2 in PC12 cells failed to produce any significant transcriptional inhibition and in this instance Bcl-2 was exclusively localized in the non-nuclear fraction. Taken together, these results strongly suggest that Bcl-2 needs to be localized at the nuclear membrane to affect transcription.

Other studies in the past have described effects of exogenously expressed Bcl-2 on NFκB activity. However, the results from these reports are controversial. Whereas some groups reported a positive effect of Bcl-2 on NFκB activity (40–43), others showed a decrease in NFκB activity following Bcl-2 overexpression (18, 44, 45). Hour et al. (44) describe a direct physical interaction between Bcl-2 and the p50 subunit of NFκB, suggesting a specificity of this effect to NFκB only. Our results suggest a more generalized effect of Bcl-2 on transcription, beyond simple interaction with an NFκB subunit. This idea is supported by additional reports in the literature, describing a negative effect of Bcl-2 not only on NFκB but also on NFAT (46–48) and p53 (20). The latter report further explores the effect of Bcl-2 mutants on p53 activity; specifically Frosch et al. (20) demonstrate the importance of membrane anchoring of Bcl-2 for it to affect p53 transactivation. This study, however, does not show any differences between various subcellular localizations of Bcl-2.

Our results introduce the novel idea that localization of Bcl-2 to specific subcellular compartments is a significant factor modulating the diverse effects of Bcl-2. Recent evidence revealing the existence of precise mechanisms responsible for proper subcellular redistribution of Bcl-2 supports this idea (24). This report describes FKBP38, an inherent calcineurin inhibitor belonging to the FK506-binding protein family, as a carrier protein responsible for redistributing Bcl-2 to its proper subcellular localization at the mitochondria, where Bcl-2 acts as an anti-apoptotic protein (49–51). The same authors reported that suppression of FKBP38 expression by small interfering RNA led to improper localization of Bcl-2, notably including the nuclear membrane, and consequently abolished its anti-apoptotic effects. When we overexpressed FKBP38 in PC12 cells transiently transfected with Bcl-2 and the SEAP-NFκB reporter gene construct that we observed in the presence of FKBP38 completely abolished the effects on NFκB-promoted transactivation of Bcl-2 and concomitantly removed Bcl-2 from the nuclear compartment. These results further suggest that nuclear localization of Bcl-2 is associated with the effects on transcription factor activity on Bcl-2.

Proper subcellular distribution of Bcl-2 to the mitochondrion requires the presence of FKBP38 (24). Our results suggest that high expression of Bcl-2, as modeled by transient but not stable transfection in vitro, leads to the appearance of Bcl-2 at the nucleus and concomitantly affects transcription factor activity. Our results also show that simultaneous expression of elevated levels of FKBP38 effectively reverses both nuclear localization and transcriptional effects of transiently transfected Bcl-2. A possible explanation for the occurrence of nuclear Bcl-2 after up-regulation of Bcl-2 expression may lie in the necessity of FKBP38 to shuttle Bcl-2 to the mitochondria. Namely, if the levels of Bcl-2 exceed the carrying capacity of FKBP38, a significant fraction of the unbound Bcl-2 would localize within the nuclear compartment (24), where Bcl-2 would exert its effects on transcription factor activity. Indeed, we found that the level of expression of transfected Bcl-2 in PC12 was lower in cells stably transfected as compared with cells transiently transfected and that no effect on transcription factor activity was exerted by Bcl-2 in the former. Whether this phenomenon reflects the possibility that nuclear Bcl-2 is incompatible with long term survival of cells and that therefore cells expressing high levels of Bcl-2 (and consequently displaying nuclear Bcl-2) were selected against during the establishment of stable transfectant cell lines remains to be established.

Nonetheless, we previously showed that suppression of the NFκB activity is incompatible with PC12 cell survival (52) and that inhibition of NFκB in the central nervous system of rats induces apoptosis in vivo (53). We also showed that in the central nervous system of aged or oxidatively stressed rats there is a free-radical dependent up-regulation of Bcl-2 expression leading to nuclear localization of Bcl-2 (22) and accompanied by perturbations of NFκB activity, as reported by us and others (18, 30, 44). It is therefore reasonable to speculate that nuclear Bcl-2, and the consequent decrease in transcription factor activity, may be part of a mechanism of cell stress response to oxidative challenges aimed at protecting cells and that nuclear Bcl-2 may contribute to elimination of those cells that failed to adapt.

To investigate possible mechanisms of action through which Bcl-2 inhibits transcription factor activity, we focused once more on NFκB as a prototypical and ubiquitous transcription factor. More importantly, the pathway leading to NFκB activation in the cytosol and its subsequent translocation to the nuclear compartment has been clearly established (37–39), thus allowing reliable result interpretation. We also used the GeneSwitch™ gene expression system in PC12 cells. This system allows rapid, homogenous, and dose-dependent gene expression following treatment with the inducing hormone MFP (Fig. 6A), thus overcoming the limitations of both transient (low transfection rate) and stable (Bcl-2 no longer localized at the nucleus) transfections. Using this system, we show that overexpressing Bcl-2 in PC12 cells by induction with 10^{-8} M mifepristone leads to a decrease in NFκB transactivation, confirming our previous results obtained in transient transfectants (30) (Fig. 6B). Subcellular fractionation followed by Western blotting analysis (Fig. 6C) show about equal distribution of Bcl-2 between the nuclear and non-nuclear compartment of the cells, also confirming our results previously obtained in transient transfectants. Having confirmed that Bcl-2 is expressed in the nuclear compartment and inhibits NFκB transactivation after induction by MFP, we wanted to investigate at which step of the pathway leading to NFκB activation and nuclear translocation Bcl-2 affects NFκB activity. NFκB is most commonly found as a p50-p65 dimer bound to inhibitory subunits of the IκB family in the cytosol of live cells (37). When proper signals hit the cells, such as stress or inflammation, IκB proteins are ubiquitinated and degraded, relieving the inhibition from the NFκB subunits and allowing them to translocate to the nucleus to bind and activate promoters of various genes (37–39).

NFκB could be inhibited at many points in this pathway, namely 1) prevention of IκB degradation and sequestration of inactive NFκB in the cytosol, 2) activation of NFκB, but prevention of its translocation to the nucleus, and 3) activation of NFκB, translocation to the nucleus, but prevention of its DNA binding. It has been previously shown that Bcl-2 can bind to the p50 subunit of NFκB within the nucleus and thereby prevent its binding to the DNA (18, 44). Whereas this evidence was limited to NFκB, it was also obtained in cells stably transfected with Bcl-2, a condition that we found generally ineffective, and therefore it cannot be excluded that it may represent an im-
Nuclear Bcl-2 Inhibits Transcription Factor Activity

I and after induction of Bcl-2 expression. Our results show that NF nuclear membrane might be affecting nuclear trafficking of DNA binding of nuclear p50 and p65. Rather, our results of induced cells, it is unlikely that Bcl-2 acts by preventing Because p50 and p65 levels are decreased in the nuclear fractions of induced and hence NF54478

transcription, possibly beyond physical interaction with one translated that Bcl-2 must be exerting a general inhibitory effect on the pMKIT Neo-mBcl-2 Dr. S. Maym University of Florida, Gainsville, FL, for the kind gift of Branch, Galveston, TX, for the kind gift of the Angiotensin-luciferase Comprehensive Cancer Center, Jacksonville, FL, for the kind gift of the pMKIT Neo-mBcl-2 expression vector. We also thank Bonnie Walters for clerical support in manuscript preparation.

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Cynthia A. Massaad, Bryce P. Portier and Giulio Taglialetela

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