Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Correlation Between Structure of Bcl-2 and Its Inhibitory Function of JNK and Caspase Activity in Dopaminergic Neuronal Apoptosis

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Abstract: To examine the correlation between the structure of Bcl-2 and its inhibitory function of c-Jun N-terminal kinase (JNK) and caspase activity, we established a dopaminergic neuronal cell line, MN9D overexpressing Bcl-2 (MN9D/Bcl-2) or its structural mutants. The mutants comprised a point mutation in the BH1 (G145A; MN9D/BH1) or BH2 (W188A; MN9D/BH2) domain and a deletion mutation in the C-terminal (MN9D/C22), BH3 (MN9D/BH3), or BH4 (MN9D/BH4) domain. As determined by the TUNEL (terminal deoxynucleotidyltransferase nick end-labeling) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay, apoptotic death of MN9D/Neo cells reached 80–90% within 24 h in response to 1 μM staurosporine. Upon staurosporine treatment, JNK activity increased six- to sevenfold over the basal level within 2–4 h. Treatment of MN9D/Neo with both staurosporine and a caspase inhibitor, Z-VAD, attenuated cell death without suppressing JNK activation. Both staurosporine-induced cell death and JNK activation were attenuated in MN9D/Bcl-2. As determined by cleavage of poly(ADP-ribose) polymerase into 85 kDa, Bcl-2 blocked caspase activity as well. When cells overexpressing one of the Bcl-2 mutants were treated with staurosporine, death was attenuated in MN9D/BH1, MN9D/BH2, and MN9D/C22 but not in MN9D/BH3 and MN9D/BH4. Similarly, both JNK and caspase activation were blocked in MN9D/BH1, MN9D/BH2, and MN9D/C22, whereas they were not suppressed in MN9D/BH3 and MN9D/BH4. Taken together, our data indicate that there exists a close structural and functional correlation of Bcl-2 to JNK and caspase activity in staurosporine-induced dopaminergic neuronal cell death.
Key Words: Apoptosis—Bcl-2 homology domain—MN9D—Parkinson's disease.
J. Neurochem. **74,** 1621–1626 (2000).

Apoptosis, also known as programmed cell death, is a highly regulated process by which an organism activates an intracellular death program and eliminates unnecessary, aged, or damaged cells (Wyllie, 1993). Apoptotic cells shrink and are rapidly taken up by neighboring cells before they elicit an inflammatory response. Given an abnormal internal and/or external proapoptotic signal(s), cells can undergo aberrant control of the positive and negative regulators of cell death, resulting in various pathological conditions including neurodegenerative disorders (Thompson, 1995).

Among many proposed mechanisms, activation of CED-3/ICE (interleukin-1-β-converting enzyme)-like proteases, recently termed caspases, seems to be a key element in the effector phase of apoptotic cell death in many paradigms (Alnemri et al., 1996; Cryns and Yuan, 1998). Once activated by a proteolytic cascade in response to proapoptotic signals, caspases kill cells by cleaving the specific cellular target proteins, using a variety of strategies (Kaufmann et al., 1993; Lazebnik et al., 1994; Cryns and Yuan, 1998). One of several mammalian mitogen-activated protein kinases, c-Jun N-terminal kinase (JNK), has been also demonstrated to mediate an early intracellular signaling pathway, eventually leading to apoptosis upon exposure to a variety of stresses (Ham et al., 1995; Xia et al., 1995; Verheij et al., 1996).

Received July 19, 1999; revised manuscript November 18, 1999; accepted November 18, 1999.

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Abbreviations used: CCM, complete culture medium; JNK, c-Jun N-terminal kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end-labeling; Z-VAD, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

Although the signaling apparatus involved in activation of the caspase remains to be further delineated, JNK signaling has been linked to both caspase-dependent and -independent cell death pathways (Muhlenbeck et al., 1998; Zanke et al., 1998).

Numerous studies have demonstrated that the 26-kDa membrane-associated protein Bcl-2 is a prototypic negative regulator of apoptosis and necrosis in some cases as demonstrated both in vivo and in vitro (Reed, 1994; Kane et al., 1995; Choi et al., 1999). Until now, 15 death-inducing and death-inhibitory members of the Bcl-2 family have been identified in mammalian cells (Adams and Cory, 1998). All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most members of the Bcl-2 family possess a carboxy-terminal membrane anchorage domain (Kroemer, 1997). Although these domains seem to be involved in determining the fate of cells upon stresses, it remains to be elucidated how these domains participate in regulating the cell death process and whether there exists cell-type and/or stress-type specificity.

Recently, we have demonstrated that (1) Bcl-2 blocks staurosporine-induced neuronal apoptotic cell death (Oh et al., 1997) and (2) Bcl-2 disrupts a signaling cascade leading to JNK activation induced by various apoptotic stresses including staurosporine (Park et al., 1997). In the present study, we have used a dopaminergic neuronal cell line, MN9D overexpressing Bcl-2 and its structural mutants, to investigate a close correlation between the structure of Bcl-2 and its function to inhibit JNK and caspase activity in staurosporine-induced neuronal cell death.

MATERIALS AND METHODS

Establishment of stable cell lines

MN9D cells were cultured in 90% Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) in an atmosphere of 10% CO_2. For establishing stable cell lines, MN9D cells plated at $\sim 2 \times 10^6$ cells onto a 25 μg/ml poly-D-lysine-coated six-well plate (Corning) were transfected with a cytomegalovirus promoter/enhancer-driven eukaryotic expression vector, pCMV/Neo, containing a full-length human Bcl-2 cDNA (MN9D/Bcl-2) or one of the several Bcl-2 mutant cDNAs using Lipofectamine (Gibco), as previously described (Oh et al., 1998). Structural mutants included Bcl-2/BH1 (G145A; MN9D/BH1), Bcl-2/BH2 (W188A; MN9D/BH2), Bcl-2/C22 (carboxy-terminal 22-amino acid deletion mutant; MN9D/C22), Bcl-2/BH3 (BH3 domain deletion mutant; MN9D/BH3), and Bcl-2/BH4 (BH4 domain deletion mutant; MN9D/BH4). MN9D cells transfected with a vector without these sequences were used as control (MN9D/Neo). These transfectants were selected in a medium containing 90% Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 500 μg/ml G-418 (complete culture medium; CCM) for 7–10 days. Colonized clones were picked, expanded in CCM, and characterized by immunoblot analysis as described below.

Immunoblot analysis and immunocytochemistry

For measuring the expression levels of Bcl-2 and its structural mutant proteins, cells ($>5 \times 10^6$) cultured on poly-D-lysine-coated P-100 dishes (Corning) were washed with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 50 m*M* Tris (pH 7.0)/2 m*M* EDTA/1% Triton X-100/2 m*M* phenylmethylsulfonyl fluoride/50 μg/ml aprotinin for 15 min. Lysates were microcentrifuged at 13,000 *g* for 30 min at 4°C. After measurement of the protein content of the resulting supernatants using a Bio-Rad protein assay kit, 50 μg of protein from each sample was separated on a 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel, blotted onto prewet polyvinylidene difluoride–nitrocellulose filters, processed for immunoblot analysis, and then developed using an enhanced chemiluminescence kit (Amersham). Rabbit polyclonal anti-Bcl-2 was used as primary antibody (1:3,000; Krajewski et al., 1993). For detecting the cleavage pattern of poly(ADP-ribose) polymerase (PARP) following staurosporine treatment, mouse monoclonal anti-PARP was used as primary antibody (1;5,000; Enzyme Systems Products; Kim et al., 1999). For immunocytochemistry, cells were fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. Subsequently, cells were incubated with a hamster monoclonal anti-Bcl-2 (6C8; a generous gift from Dr. S. J. Korsmeyer at Washington University) followed by fluorescein isothiocyanate-conjugated goat anti-hamster IgG and examined using a Carl Zeiss Axiovert 100 equipped with epifluorescence. For in situ labeling of nuclear DNA fragmentation, terminal deoxynucleotidyltransferase-mediated nick end-labeling (TUNEL) assay was carried out as previously described (Gavrieli et al., 1992). Bright-field photomicrographs were taken under a Carl Zeiss Axioplan2 microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

Approximately 2×10^4 cells of each stable cell line were plated on a 25 μg/ml poly-D-lysine-coated 48-well plate (Corning), cultured in CCM for 2 days, and switched to serum-free N2 medium with or without 1 μ*M* staurosporine. If necessary, a broad-spectrum caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD; 200–400 μ*M*) was applied with staurosporine. Following drug treatment for 24 h, the rate of cell survival was measured using colorimetric MTT reduction assay as described previously (Hansen et al., 1989; Oh et al., 1997). Formazan grains formed by dehydrogenase activity within the surviving cells were solubilized with 20% SDS in 50% aqueous dimethylformamide overnight. The optical density of the dissolved grains was assessed at 540 nm using an ELISA plate reader (Molecular Devices). Values from each treatment were expressed as a percentage of survival over the untreated matching control (100% survival).

JNK assay

MN9D cells from each transfectant were treated with 1 μ*M* staurosporine with or without 200 μ*M* Z-VAD for the indicated time periods, washed twice with cold PBS/EDTA buffer, and lysed on ice for 15 min in 1% Nonidet P-40/20 m*M* HEPES (pH 7.4) 100 m*M* NaCl/20 m*M* β-glycerophosphate/0.2 m*M* sodium vanadate/1 m*M* EDTA/1 m*M* phenylmethylsulfonyl fluoride/50 μg/ml aprotinin. After microcentrifugation, quantified amounts of the resulting supernatants were immunoprecipitated with mouse monoclonal anti-JNK1 antibody (Pharmingen). The immunopellets were assessed for JNK1 activity as described previously (Park et al., 1997). Glutathione *S*-transferase–c-Jun fusion protein was used as a substrate. The reaction mixtures were subjected to electrophoresis on a 10% SDS–polyacrylamide gel. Following autoradiography, the den-

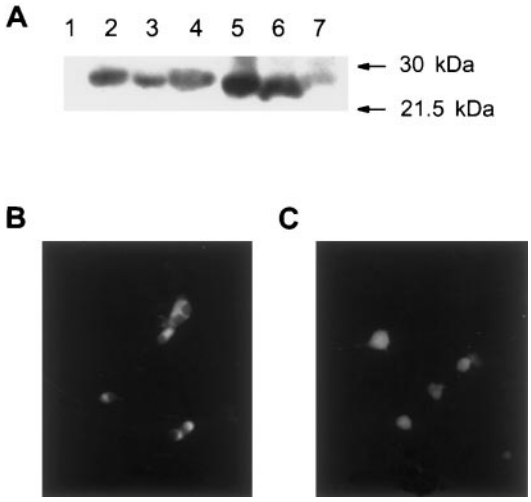

FIG. 1. Characterization of MN9D cells overexpressing Bcl-2 and its structural mutant proteins. **A:** Immunoblot analysis of Bcl-2 and its mutant proteins in MN9D/Neo (lane 1), MN9D/Bcl-2 (lane 2), MN9D/C22 (lane 7), MN9D/BH1 (lane 3), MN9D/BH2 (lane 4), MN9D/BH3 (lane 5), and MN9D/BH4 (lane 6) cells. Rabbit polyclonal anti-Bcl-2 was used as the primary antibody (1:3,000; Krajewski et al., 1993). Arrows indicate 30-kDa and 21.5-kDa molecular size markers, respectively. **B** and **C:** Immunofluorescent localization of Bcl-2 was carried out in MN9D/Bcl-2 (B) and MN9D/C22 (C) cells. For localization, a hamster monoclonal anti-Bcl-2 antibody (1:100, 6C8) was followed by fluorescein isothiocyanate-conjugated goat anti-hamster IgG.

sity of the phosphorylated proteins was quantitated by an image analyzer (Fuji).

RESULTS

To establish the stable dopaminergic neuronal cell line, MN9D overexpressing wild-type human Bcl-2 or one of its structural mutant proteins, cells were transfected with a cytomegalovirus major immediate early enhancer/promoter-driven eukaryotic expression vector containing a full-length human Bcl-2 or its structural mutant cDNAs. Among randomly picked 12–24 G-418-resistant clones from each transfectant, clones that expressed higher levels of equivalent mRNA were chosen for further characterization by immunoblot analysis. As shown in Fig. 1A, the endogenous level of Bcl-2 in control MN9D/Neo cells was not readily detectable, whereas all Bcl-2 transfectants demonstrated high levels of wild-type Bcl-2 or its equivalent mutant proteins. As demonstrated in Fig. 1B and C, a diffuse staining over the whole cytosol and nuclei in MN9D/C22 cells was distinctly different from that in the other Bcl-2 transfectants.

When MN9D/Neo cells were first treated with 1 μM staurosporine, dying cells exhibited TUNEL-positive nuclei typical of apoptosis compared with untreated MN9D/Neo cells (Fig. 2A and B). This was consistent with our previous studies in that staurosporine largely induces apoptotic death in MN9D cells (Oh et al., 1997; Kim et al., 1999). As determined by MTT reduction assay, ~89.6% of MN9D/Neo cells were dead upon exposure to staurosporine for 24 h (Fig. 2C). This was largely attenuated in the presence of Z-VAD, suggesting that caspase activation is involved. To evaluate the functional role and requirement of structural motifs of Bcl-2 in sparing MN9D cells from staurosporine-induced cell death, MN9D cells overexpressing Bcl-2 and its structural mutants were then treated with 1 μM staurosporine for 24 h. Staurosporine-induced cell death was significantly attenuated in MN9D/Bcl-2 cells (20.1% cell death). The majority of MN9D/Bcl-2 cells were morphologically intact, albeit round (not shown). As shown in Fig. 2C, MN9D/BH1, MN9D/BH2, and MN9D/C22 cells were equally as protected as MN9D/Bcl-2 cells upon staurosporine treatment. In contrast, staurosporine killed both MN9D/BH3 and MN9D/BH4 cells. The rate of cell death in these two transfectants was quite comparable with that in MN9D/Neo cells. Similar patterns were obtained from at least two to three independently derived clones from each transfectant (not shown).

It has been reported that the JNK pathway plays an important role in the regulation of neuronal apoptosis (Ham et al., 1995; Virdee et al., 1997). As shown in Fig. 3A, treatment of MN9D/Neo cells with 1 μM staurospor-

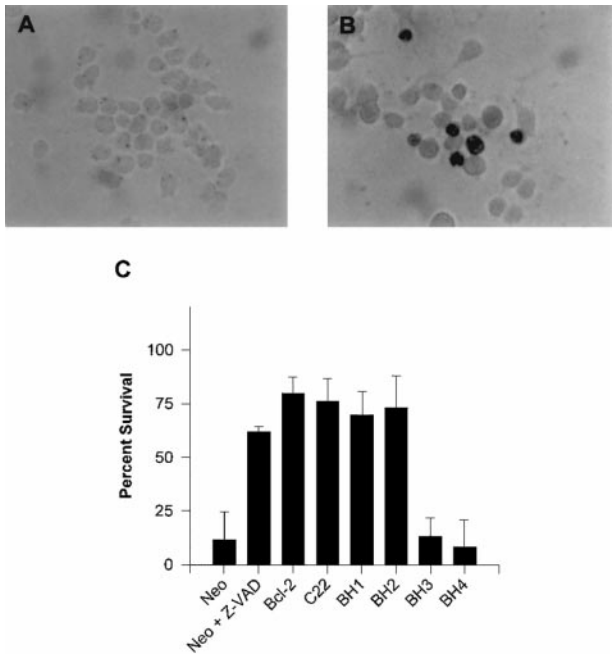

FIG. 2. Functional role of Bcl-2 and its structural mutant proteins in staurosporine-induced MN9D cell death. TUNEL assay was carried out in MN9D/Neo cells following treatment with a vehicle (**A**) or 1 μM staurosporine (**B**) for 12 h. Cells from each transfectant were plated at 2×10^4 on a poly-D-lysine-coated 48-well plate, cultivated in CCM for 2 days, and switched to N2 medium with or without 1 μM staurosporine for 20–24 h (**C**). If necessary, 200 μM Z-VAD (a broad-spectrum caspase inhibitor) was added. Subsequently, viability was measured by the MTT reduction assay. Values from each treatment were expressed as a percent over the untreated control (100%). Each column represents the mean ± SEM from three independent experiments in triplicate.

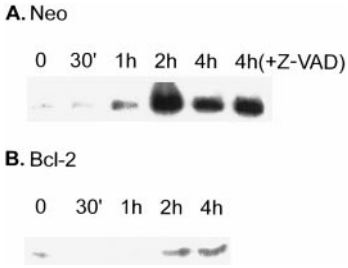

FIG. 3. JNK assay following staurosporine treatment in MN9D/Neo (**A**) and MN9D/Bcl-2 (**B**) cells. Cells from MN9D/Neo and MN9D/Bcl-2 were plated at 1–2 $\times$ 10^6 on poly-D-lysine-coated P-100 Petri dishes, cultivated in CCM for 2 days, switched to N2 medium containing 1 μM staurosporine with or without 200 μM Z-VAD (a broad-spectrum caspase inhibitor) for the indicated time periods, and then subjected to the JNK assay as previously described (Park et al., 1997).

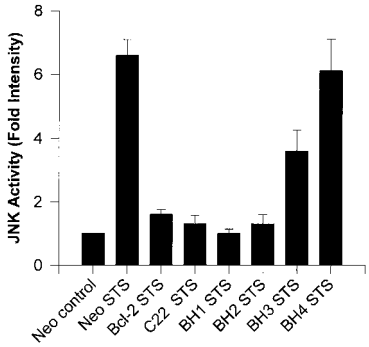

FIG. 4. Regulation of JNK activity by Bcl-2 structural mutants. Cells from MN9D/Neo, MN9D/Bcl-2, MN9D/C22, MN9D/BH1, MN9D/BH2, MN9D/BH3, and MN9D/BH4 were plated at 1–2 $\times$ 10^6 on poly-D-lysine-coated P-100 Petri dishes, cultivated in CCM for 2 days, switched to N2 medium containing 1 μM staurosporine (STS) for 2 h, and then subjected to the JNK assay. The intensity of the phosphorylated glutathione *S*-transferase–Jun was quantitated by phosphoimage analyzer. Values were expressed as fold intensity over the untreated matching control. Data represent the means $\pm$ SD from three or four independent experiments.

ine activated the JNK signaling pathway. Following staurosporine treatment, activation of JNK started as early as 30 min and continuously increased thereafter up to six- to sevenfold over the basal level of untreated MN9D/Neo cells. This activation was not blocked in the presence of 200–400 μM Z-VAD, suggesting that caspase activation is downstream of JNK activation. In MN9D/Bcl-2 cells, staurosporine had a minimal effect on JNK activity (Fig. 3B).

To investigate whether there exists a correlation between the requirement of Bcl-2 structural motifs for protection against staurosporine-induced cell death and the blockage of JNK and caspase activation, each transfectant was treated with 1 μM staurosporine for 2 h and subjected to an assay for JNK activity. As shown in Fig. 4, activation of JNK activity was suppressed in MN9D/BH1, MN9D/BH2, and MN9D/C22 cells following staurosporine treatment. The rate of suppression in these transfectants was quite similar to that in MN9D/Bcl-2 cells. In contrast, the rate of JNK activation upon staurosporine treatment in MN9D/BH3 and MN9D/BH4 cells increased up to 3.7–6.2-fold over the untreated matching control, which was comparable with that in MN9D/Neo cells. When the cleavage pattern of PARP by activated caspase was subsequently examined by immunoblot analysis (Kaufmann et al., 1993), specific cleavage of PARP into the 85-kDa form was detected following treatment with 1 μM staurosporine as early as 6 h in MN9D/Neo cells and continued up to 24 h (Fig. 5). The formation of the 85-kDa band was seen in MN9D/BH3 and MN9D/BH4 cells, whereas it was not detected in MN9D/Bcl-2, MN9D/BH1, MN9D/BH2, or MN9D/C22 cells up to 24 h (Fig. 5).

DISCUSSION

Previously, we have demonstrated that suppression of the JNK signaling pathway in N18TG2 neuroglioma cells may be critical for the protective action of Bcl-2 against various apoptotic stresses including staurosporine (Park et al., 1997). The data presented here extend our previous observations by demonstrating that overexpression of Bcl-2 in mesencephalon-derived MN9D dopaminergic neuronal cells protects against staurosporine-induced cell death by inhibiting JNK activation and its downstream caspase activation as well. More importantly, we have first demonstrated that there exists a close correlation between the structure of Bcl-2 and its function to inhibit JNK and caspase activity. In this cell death paradigm, subcellular localization of Bcl-2 may not be critical in that deletion of the carboxy-terminal membrane-anchorage domain of Bcl-2 does not affect the suppression rate of JNK and caspase activity or its antiapoptotic role. Similarly, we have also found that MN9D cells overexpressing BH1 or BH2 point mutant protein are equally protective. These data suggest that the known hetero- and homodimerization domains among Bcl-2 family proteins may not be as important as previously thought for the regulation of apoptosis in MN9D cells. As previous findings indicate that BH3 and BH4 do-

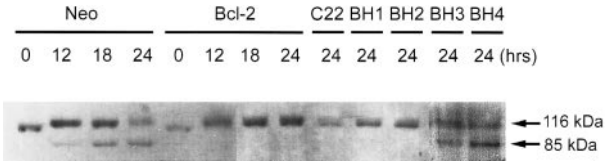

FIG. 5. Regulation of caspase activity by Bcl-2 structural mutants as determined by immunoblot analysis for PARP. Cells from MN9D/Neo, MN9D/Bcl-2, MN9D/C22, MN9D/BH1, MN9D/BH2, MN9D/BH3, and MN9D/BH4 were plated at 1–2 $\times$ 10^6 on poly-D-lysine-coated P-100 Petri dishes, cultivated in CCM for 2 days, and switched to N2 medium containing 1 μM staurosporine for the indicated time periods. Cells were lysed and processed for immunoblot analysis as described in Materials and Methods. Specific bands of PARP and its cleaved form were detected with a mouse monoclonal anti-PARP antibody (1:5,000).

mains are necessary for Bcl-2 to interact with death-regulatory proteins (H. G. Wang et al., 1996; K. Wang et al., 1996; Huang et al., 1998), deletion of these domains abrogates the death-inhibitory function of Bcl-2 in staurosporine-induced MN9D cell death. Based on our present data, at least one of the plausible mechanisms by which BH3 and BH4 influence the fate of cell death could be ascribed to its capacity to suppress JNK signaling and its downstream caspase activition in MN9D cells.

Earlier studies indicate that hetero- and homodimerization through BH1 and BH2 are important for the regulation of cell death by Bcl-2 family proteins (Oltvai et al., 1993; Yin et al., 1994). Through BH1 and BH2 domains, Bcl-2 can, for example, heterodimerize with the proapoptotic protein Bax, and the ratio of Bcl-2 to Bax determines survival or death following various apoptotic stimuli. Therefore, substitution of Gly^{145} in the BH1 domain or Trp^{188} in the BH2 domain completely abrogates the death-repressor activity of Bcl-2 in various apoptotic cell death paradigms (Yin et al., 1994). Contrary to these early indications, others have recently suggested that heterodimerization through the BH1 and BH2 domain is not required for antiapoptotic function (Cheng et al., 1996; Kelekar et al., 1997). In our laboratory, we have observed that overexpression of the BH1 or BH2 point mutant Bcl-2 protein in PC12 cells does not prevent staurosporine-induced apoptosis (I. I. Chang et al., unpublished data). These observations from PC12 cells are quite contrary to our present finding that MN9D cells overexpressing one of these point mutant proteins can render protection against staurosporine-induced cell death by effectively suppressing JNK and caspase activity. Interestingly, nigericin (potassium/proton ionophore)-induced caspase activation and subsequent cell death are effectively blocked in both MN9D/BH1 and MN9D/BH2 cells as well (J. H. Oh et al., unpublished data). Although it is speculative at present, these contradictory data may therefore raise the possibility that the requirement of these dimerization domains of Bcl-2 may be cell and/or stress type specific.

Most members of the Bcl-2 family possess a carboxy-terminal transmembrane domain, but others including Bid and Bad do not (Kroemer, 1997). It has been demonstrated that the carboxy-terminal hydrophobic domain of Bcl-2 is important in docking at the nuclear, outer mitochondrial, and endoplasmic reticular membranes (Nguyen et al., 1993, 1994; Oltvai et al., 1993). Anchoring Bcl-2 to these strategic membrane locations and its concentration at these sites in the cells seem to contribute to its proposed antiapoptotic function (Nguyen et al., 1994). Consequently, the ability of Bcl-2 to suppress apoptosis has been demonstrated to abrogate or diminish in constructs that lack carboxy-terminal transmembrane domain or in constructs in which the single predicted transmembrane domain is replaced by the corresponding domain of a plasma membrane protein (Alnemri et al., 1992; Hockenbery et al., 1993; Tanaka et al., 1993). Similarly, fusion of a heterologous transmembrane domain to Bcl-2 protein that lacks the carboxy-terminal domain restores antiapoptotic function (Tanaka et al., 1993). It has been suggested that cytochrome *c* release from the mitochondria to cytosol eventually activates effector caspases, leading to apoptotic cell death in response to stresses including staurosporine (Kluck et al., 1997; Yang et al., 1997). Based on these findings, it has been proposed that anchored Bcl-2 at the outer membrane of mitochondria may act to inhibit translocation of cytochrome *c* and subsequent caspase activation in the apoptotic process. However, a deletion of the carboxy terminal does not abrogate the inhibitory effect of Bcl-2 on staurosporine-induced JNK and caspase activation in MN9D cells. Our present data are consistent with another finding demonstrating that a carboxy-terminal truncation mutant of Bcl-2 shows full activity in blocking apoptosis of nerve growth factor-deprived sympathetic neurons and tumor necrosis factor-α-treated fibroblasts (Borner et al., 1994). Therefore, it may be that the requirement of the membrane anchorage domain of Bcl-2 also depends on cell type and/or stress type.

Although the mechanism of apoptosis is proposed to be remarkably conserved, the expected greater complexity and diversity in mammals seem to exist. For example, it appears that the structure–function requirement of the Bcl-2 family may be more complicated than thought before. Further studies delineating the cell type and/or stress type specificity will expand our present understanding of how cell death-regulatory proteins such as the Bcl-2 family and other structurally unrelated proteins interact to modulate the dynamic equilibrium of cell life and death.

Acknowledgment: We gratefully acknowledge Drs. A. Heller and L. Won (University of Chicago) for providing us with the MN9D cell line. This work is supported by funds from KOSEF through the Brain Disease Research Center at Ajou University and STEPI (to Y.J.O.) and by the Brain Research Program for MOST (to T.H.O.).

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