bcl-X S -induced Cell Death in 3T3 Cells Does Not Require or Induce Caspase Activation 1

Jordan S. Fridman, Mary A. Benedict, and Jonathan Maybaum 2

Departments of Pharmacology [J. S. F., J. M.] and Pathology [M. A. B.], University of Michigan Medical School, Ann Arbor, Michigan 48109

ABSTRACT

Using a tetracycline-regulated expression system, we have shown that expression of bcl-X S is sufficient to induce acute cell death in 3T3 cells, and that the manner in which these cells die is both morphologically and biochemically different from Fas/CD95-induced apoptosis. bcl-X S expression causes loss of the inner mitochondrial membrane potential (ΔΨm) but does not induce caspase activation. Loss of viability, as determined by mitochondrial function and ethidium bromide exclusion, was not inhibited by the broad-spectrum caspase inhibitor zVAD-fmk or by expression of a dominant negative caspase 9 (DN). However, zVAD-fmk was efficacious in inhibiting cell death triggered by an activating anti-Fas/CD95 antibody. In addition, bcl-X S does not possess the 5th and 6th α-helices (thought to be the membrane-spanning domains in bcl-2, bcl-X L , and bax) and, therefore, should not be able to form membrane channels, thus eliminating this possible mechanism of action. The finding that bcl-X S kills 3T3 cells without caspase activation, along with the absence of membrane spanning domains in bcl-X S , may, therefore, represent a novel cell death pathway for the pro-death bcl-2 family members.

INTRODUCTION

Members of the bcl-2 family of genes serve as regulators of cell death, either promoting (Bax, Bak, Bok, Bik, Hrk, Bad, Bid, Diva, and EGL-1) or inhibiting it (Bcl-2, Bcl-X L , Bcl-w, Mcl-1, and CED-9). How these proteins function is still unclear but redundancy and multiple mechanisms for each member seem to be the norm rather than the exception. In addition, some of these proteins appear to exhibit qualitatively different properties, depending on the context in which they are expressed. However, despite these variations, the current models that are proposed to explain their actions converge on three main events: (a) formation of homo- or heterodimers within the bcl-2 family; (b) binding to proteins outside of the bcl-2 family; and (c) the formation of membrane-spanning channels (see Refs. 1 and 2 for review).

Among the proapoptotic bcl-2 family members, bcl-X S is unique in that it lacks BH1 and BH2 domains, which constitute part of the proposed membrane spanning-domains of bcl-X L , bcl-2, and bax (3–6), whereas it possess both a BH3 and a BH4 domain. The BH3 and BH4 domains enable bcl-X S to bind to bcl-X L 3 (7) and, theoretically, to unrelated proteins such as Apaf-1 (8–10). In view of this unusual combination of structural features, we thought that an exam-

MATERIALS AND METHODS

Reagents. TET-hydrochloride, ammonium persulfate, ethidium bromide, anti-HA antibody, and a protease inhibitor cocktail (Complete) were supplied by Boehringer Mannheim (Indianapolis, IN). Protein assay kits were obtained from Bio-Rad (Hercules, CA). DMEM, Lipofectamine Plus, and antibiotic solutions were purchased from Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum was bought from Clontech (Palo Alto, CA). Horseradish peroxidase-conjugated antirabbit and antimouse antibodies and SuperSignal ULTRA chemiluminescent substrate were obtained from Pierce (Rockford, IL). Rhodamine 123 and JC-1 were purchased from Molecular Probes, Inc. (Eugene, OR). Fluorogenic caspase substrates and zVAD-fmk were obtained from Enzyme Systems Products (Livermore, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), or Fisher Scientific (Cincinnati, OH).

Cell Culture, Subcloning, and Derivation of 9DN. 3T3 clonal cell lines expressing bcl-X S -HA under the control of a TETregulated retroviral expression system (3T3XS cells; Ref. 11) were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Clontech) and t-glutamine (2 mM) and were grown as monolayers at 37°C humidified with 5% CO 2 . Cells were maintained in the presence of TET at a final concentration of 1 μg/ml except where noted.

Subclones of 3T3XS cells were isolated by plating serial dilutions of cells onto tissue culture dishes and choosing small isolated colonies of cells (5–10 days later) to transfer to 24-well dishes using filter paper soaked in trypsin (Life Technologies, Inc.). Subclones were expanded and analyzed for regulated, homogenous gene expression by Western blot analysis as described previously (11).

Stable cell lines expressing a 9DN construct were made from 3T3XS7.2 cells by transfection with pCNA3-ICE-LAP6DN-HA (Ref. 8; a gift from Gabriel Nunez’s laboratory, University of Michigan, Ann Arbor, MI) using the K12 rat colon carcinoma cells, it did so without inducing significant levels of apoptosis (11). This was unexpected in view of previous evidence showing that bcl-X S expressed using an adenoviral expression vector (12–14) or by transient transfection,3 did cause significant apoptosis. This observation led us to the question of whether bcl-X S expression by itself (that is, in the absence of additional stresses of adenoviral expression or transfection) is indeed sufficient to cause a robust apoptotic response.

In this report, we show that expression of bcl-X S (in the absence of other stresses) is sufficient to elicit a swift cell death in 3T3 mouse fibroblast cells. This response was closely correlated with the loss of the mitochondrial inner membrane potential (ΔΨm), despite the fact that bcl-X S lacks the domains necessary to form channels that could directly mediate such an effect. The mechanism by which bcl-X S kills 3T3 cells is different from apoptosis caused by the activation of the Fas/CD95 system in that the former is completely independent of caspase activation. Furthermore, the activity of bcl-X S in these cells is apparently not due to induction of apoptosis through a pathway, which relies on the ability of bcl-X S to form channels because these domains (α5- and α6-helices) are absent. These findings imply that the spectrum of actions caused by bcl-X S may be unique among pro-death members of the family.

[Received 5/18/99; accepted 10/4/99.]

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3This work was supported by NIH Grant CA56663 and NIH Pharmacological Sciences Training Grant GM07767 from National Institute of General Medical Sciences.

To whom requests for reprints should be addressed, at Department of Pharmacology, University of Michigan Medical School, 4701 Upland Center, Ann Arbor, MI 48109-0504. Phone: (734) 647-1436; E-mail: maybaum@umich.edu.

4G. Nunez, M. A. Benedict, and N. Inohara, unpublished data.

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lipofectamine plus reagent (Life Technologies, Inc.) following the manufacturer's protocols. Clones were selected in media containing 500 mg/ml G418, and expression was confirmed by Western blot analysis using an anti-HA antibody. The functionality of the clones was confirmed by growing cells transfected with the pCDNA3-ICE-LAP6DN-HA construct in serum-free media and comparing their sub-2N DNA-containing population with the parental line (3T3XS7.2 cells) grown in identical conditions. pCDNA3-ICE-LAP6DN-HA expressing cells, henceforth referred to as 9DN cells, are highly resistant to serum starvation-induced death (compared with their parental cells).

Photomicroscopy. Experiments were performed on Lab-Tek Chamber Slides (Nunc, Inc.). Approximately 7 × 10^4 cells were plated onto each slide, and they were left to grow for 24–48 h (+TET). For TET withdrawal experiments, slides had their media changed to −TET at different times so that all of the time points were photographed at the same time. The medium was changed daily. Fas/Act D-treated slides were also treated at various times so that they, too, could be photographed simultaneously. Cells were maintained in TET (1 μg/ml) and the medium was changed daily. ZVAD-fmk was added to the specified slides 30 min before treatment with Fas/Act D or at the time of withdrawal from TET, and control slides were given a similar amount of DMSO (0.1%). ZVAD-fmk was replaced daily if time points were more than 24 h. Phase contrast photos were taken on a Leica DMIL microscope and slides were subsequently stained with a 25× ethidium bromide/acridine orange solution (100 μg/ml, each, ethidium bromide and acridine orange in PBS). Stained cells were then photographed using a dual-fluorochrome filter on an Olympus inverted fluorescent microscope.

Caspace Activity Assay. 3T3XS7.2 cells were plated in 150-mm tissue culture dishes (approximately 7 × 10^5) and allowed to grow for 24–48 h in the presence of TET. In the TET withdrawal time course experiment, plates were withdrawn from TET for various times (the medium was changed daily) and collected by trypsinization followed by neutralization of the trypsin with the media that the cells were growing in (to retain any floating cells). Cells were then washed twice with cold PBS, lysed with caspase assay lysis buffer (Clontech), and stored at −80°C until assayed. Fas/Act D-treated cells were plated in 100-mm dishes (approximately 4 × 10^6 cells/dish), and, after 24–48 h, they were treated with anti-Fas antibody (500 ng/ml) and Act D (4 μM). Cells that were treated with ZVAD-fmk (50 μM) had the inhibitor added 30 min prior to any treatment and then changed after 24 h. Control samples received DMSO (0.1%). Manufacturer’s protocols were followed for the assays with minor modifications. Briefly, samples were thawed on ice and were centrifuged at 4°C to pellet membranes. Supernatants were then transferred to new tubes, and their protein concentration was quantitated. Equal amounts of protein from each sample were aliquoted into microtiter plates and left on ice (volume was equalized with lysis buffer). Assay buffer [100 mM HEPES (pH 7.5), 10% sucrose (w/v), 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 10 mM DTT, and 2 μM antibody-forming cell-conjugated caspase substrate] was prepared fresh, and 50 μl was added to the appropriate wells. Plates were then read in a 96-well fluorimeter at 30°C every 5 min for 1 h. The excitation wavelength was 450 nm, and the emission wavelength was 530 nm. Maximal rates of substrate cleavage were then obtained using the accompanying software.

Detection of PARP Degradation. For TET withdrawal experiments, 3T3XS7.2 cells were plated in 150-mm tissue culture dishes (approximately 7 × 10^5) and allowed to grow for 24–48 h in the presence of TET. Plates were withdrawn from TET for various times (medium was changed daily) and collected by trypsinization followed by neutralization of the trypsin with the media in which the cells were growing (to retain any floating cells). Cells were then washed twice with cold PBS and lysed with a NP40 lysis buffer (11). Lysates were spun at 10,000 rpm, and supernatants were stored at −80°C. Fas/Act D-treated cells were plated in 100-mm dishes (approximately 4 × 10^6 cells/dish), and, after 24–48 h, they were treated with anti-Fas antibody (500 ng/ml) and Act D (4 μM). Cells were harvested and analyzed simultaneously. Each duplicate plate was harvested for Western blot analysis as described previously (11). Rhodamine 123 (Rh-123) was added from a 5 mg/ml stock (in ethanol) to the plates at a final concentration of 5 μg/ml and placed back in the incubator for 40 min. The medium was then aspirated and 4 ml of trypsin were added to each plate; the plates were then replaced in the incubator for 1–2 min. The trypsin (now containing the detached cells) was transferred to flow cytometry tubes and placed immediately on ice. Samples were then spun in a cold centrifuge, and the trypsin was aspirated. Fifty hundred μl of ice-cold PBS were then added to each tube, and the samples were left on ice in the dark until they were analyzed in the Becton Dickinson FACScan flow cytometer (5–10 min). Similar experiments were also performed using the potential dependent dye JC-1 (Molecular Probes, Inc.; Ref. 15). JC-1 was used at a final concentration of 5 μg/ml using the same protocol as with Rh-123. High ΔΨm cells were defined as those cells with high red fluorescence (FL2 channel).

RESULTS

Time Course of bcl-Xs-HA Expression in 3T3XS Cells. Two subclones, 3T3XS7.2 (clone 7.2) and 3T3XS10.2 (clone 10.2), were initially characterized for bcl-Xs expression levels and phenotypic changes after withdrawal from TET. After they were found to behave in a similar fashion, clone 7.2 was arbitrarily chosen for all of the ensuing experiments; however, clone 10.2 was used to confirm a number of key biochemical findings in the 7.2 cells. Regulated bcl-Xs-HA expression was confirmed by Western blot analysis using equal amounts of protein collected from 3T3XS7.2 cells at various times after replacement of maintenance medium (1 μg/ml TET) with medium lacking TET. Using an anti-HA antibody (Fig. 1), expression of bcl-Xs-HA was detectable beginning at 30–32 h and remained unchanged through 48 h. Similar blots were probed with an anti-bcl-X antibody, and identical results for bcl-Xs expression were obtained; bcl-Xs expression was consistently detected at a low level compared with bcl-Xs (data not shown).

Cell Death Induced by bcl-Xs in 3T3 Cells Differs Morphologically from Anti-Fas Antibody-Induced Death. Upon induction of bcl-Xs expression, 3T3XS7.2 cells die rapidly. At about 6 h after the onset of bcl-Xs expression (that is, 36 h after the removal of TET), cell death is observed, characterized by the rounding of the cells, cytoplasmic condensation, and a loss of cell-to-cell contact (Fig. 2). When we compared the morphology of cells dying from expression of bcl-Xs with the same cells (grown +TET) treated for 6 h with anti-Fas antibody (500 ng/ml) and Act D (4 μM; Fas/Act D) the dying cells looked remarkably different. The Fas/Act D cells, like their bcl-Xs-expressing counterparts, had loss of cell-to-cell contact, and cellular condensation, but their plasma membrane was also highly blebbled, resembling popcorn (Fig. 2, lower right) leading us to postulate that there are substantial differences, either qualitative or quantitative, in the death pathways activated by these stimuli.

Fig. 1. Detection of bcl-Xs-HA after withdrawal from TET (1 μg/ml). The indicated times represent the duration of growth in the absence of TET before harvesting of cells. 3T3XS7.2 cells were withdrawn from TET at various times to allow for the simultaneous collection of all of the samples. Growth medium was changed daily. Equal amounts of protein were used in Western blots as described in “Materials and Methods.” Blots were probed using an anti-HA monoclonal antibody (1 μg/ml). This experiment is representative of four experiments performed.
Fig. 2. Morphological differences between bcl-X<sub>S</sub> and Fas/Act D-induced cell death in 3T3 cells. Phase contrast micrographs of 3T3XS7.2 cells withdrawn from TET for 36 h (~6 h of bcl-X<sub>S</sub> expression, left) or treated with anti-Fas antibody (500 ng/ml) and 4 μM Act D for 6 h (Fas/Act D, right) were taken, and the highlighted areas were subsequently digitally magnified (lower left and lower right). Cells expressing bcl-X<sub>S</sub> show rounded, shrunken cells with no blebbing (lower left), and cells treated with Fas/Act D possess extensive membrane blebbing resembling popcorn (lower right). Control cells (CON) are 3T3XS7.2 cells grown in the presence of TET. This experiment is representative of at least three independent experiments. Arrows, dead or dying cells.

Similar results were obtained using the second bcl-X<sub>S</sub>-expressing clone, 3T3XS10.2, and no morphological changes were observed as the β-galactosidase-expressing cells, 3T3LZ 9.3, were withdrawn from TET (data not shown).

To examine the morphological differences more closely, we stained these same cells with a combination of acridine orange and ethidium bromide (Fig. 3). The bcl-X<sub>S</sub>-induced population had numerous cells stained red from ethidium bromide (Fig. 3). The bcl-X<sub>S</sub>-induced population had numerous cells stained red from ethidium bromide, indicating the loss of membrane integrity, but this occurs mainly after the cells have become rounded and condensed. In contrast, the Fas/Act D-treated population contained red and yellow-green cells. These changes were prevented in the Fas/Act D-treated cells by the addition of zVAD-fmk (50 μM; Fig. 3, middle right); however, zVAD-fmk (up to 100 μM) was unable to block the appearance of red-stained cells on induction of bcl-X<sub>S</sub> (Fig. 3, middle left). In addition, stable expression of 9DN in 3T3XS7.2 cells also did not prevent the morphological changes observed in 3T3XS7.2 cells on the withdrawal from TET (data not shown). As can be seen in Fig. 3, the kinetics of the two death pathways are quite different; however, both of these treatments result in the demise of nearly all of the cells within 96 h (not shown). Cells withdrawn from TET for 32–36 h or those treated with Fas/Act D for 4–6 h show equivalent amounts of cell death as determined by the population of cells possessing less than their normal DNA content (data not shown). The Fas/ActD-treated cells also lose their membrane integrity, but this occurs mainly after the cells have become rounded and condensed.

Procaspase Activation in bcl-X<sub>S</sub> and Fas/Act D-induced 3T3 Cell Death. To determine whether procaspases were being activated during bcl-X<sub>S</sub>-induced cell death, we made extracts from 3T3XS7.2 cells at various times after withdrawal from TET and assayed these extracts for their ability to cleave fluorogenic caspase substrates (Fig. 4). We also isolated cellular extracts from 3T3XS7.2 cells grown in the presence of TET and treated with a combination of anti-Fas antibody (500 ng/ml) and Act D (4 μM; Fas/Act D). In response to Bcl-X<sub>S</sub>-HA expression, there was no detectable increase in CPP-32/caspase 3-like (DEVD), ICE-like (YVAD), or caspase 9-like (LEHD) activities. These results were confirmed in the 3T3XS10.2 cells as well as in the control cell line 3T3LZ9.3 (data not shown). CPP32/caspase 3 activation was also assessed by Western blot analysis using anti-CPP-32 antibodies that detect either the Mr 32,000 species or the activated p10 cleavage product, again, no activation was detected in bcl-X<sub>S</sub>-expressing cells (data not shown). There was, however, a large increase in DEVDase activity and a minor increase in LEHDase activity that were detectable 6 h after 3T3XS7.2 cells were treated with Fas/Act D. Therefore, although 3T3XS7.2 cells are capable of dying through a caspase-mediated pathway, such activities are not activated or required for bcl-X<sub>S</sub>-induced death in 3T3XS7.2 cells.

Degradation of PARP on Expression of bcl-X<sub>S</sub> or Treatment with Fas/Act D. We next examined PARP, the degradation of which is considered to be a marker of caspase activity. PARP is a Mr 116,000 nuclear chromatin-associated enzyme that catalyze the transfer of ADP-ribose from NAD<sup>+</sup> to numerous proteins in the nucleus (reviewed in Ref. 16). PARP was partially degraded after 36 h of TET withdrawal (Fig. 5). Degradation was also seen after 24 h of treatment with Fas/Act D but not at the 6-h time point, at which time there was substantial DEVDase activity (Fig. 4). Therefore, we conclude that PARP degradation, on expression of bcl-X<sub>S</sub>, occurs by the activation of an unidentified protease that lacks caspase 1-, 3-, or 9-like activities.

Loss of Mitochondrial Inner Membrane Potential (ΔΨm) Occurs Coincidentally with Bcl-X<sub>S</sub> Expression and Is Not Inhibited by zVAD-fmk or 9DN. After concluding that bcl-X<sub>S</sub> does not require caspases to kill 3T3 cells, we next looked at mitochondrial effects that have been suggested to be upstream of caspase activation. Others have shown that the BH3 domain-containing pro-death bcl-2 family members can cause the loss of ΔΨm. Therefore, we used rhodamine 123 and flow cytometry to measure the ΔΨm as TET was withdrawn from 3T3XS7.2 cells in the absence (Fig. 6A) or presence (Fig. 6B) of zVAD-fmk as well as the ΔΨm of the 9DN cells with or without TET (Fig. 6C). A large decrease in the ΔΨm was detectable 32 h after withdrawal from TET (in this representative experiment), and Western blot analysis of duplicate culture plates showed a concomitant decrease in the Mr 116,000 species or the activated p10 cleavage product, again, no activation was detected in bcl-X<sub>S</sub>-expressing cells (data not shown). The 9DN derivative of 3T3XS7.2 cells had a ΔΨm identical to the parental cells in the presence of TET and a loss of the ΔΨm that was indistinguishable from that of the 3T3XS7.2 cells after TET withdrawal (Fig. 6C). Loss of the ΔΨm was shown not be an artifact of the expression system because the 3T3LZ 9.3 cells showed no loss of ΔΨm through 48 h after TET withdrawal (data not shown). Therefore, bcl-X<sub>S</sub> can cause loss of the ΔΨm and cell death without zVAD-fmk-sensitive proteases or a functional caspase 9 pathway. These experiments were repeated using another fluorescent dye, JC-1 (17), to rule out the possibility of artifacts from the use of rhodamine 123, and similar results were obtained (data not shown).

DISCUSSION

In this report, we have shown that bcl-X<sub>S</sub> expression is sufficient to induce acute cell death in 3T3 cells and that the manner in which these
cells die is morphologically and biochemically different from CD95/Fas-induced cell death. Caspase activity was not detected in populations of cells dying from enforced expression of bcl-X<sub>S</sub>, and cell death is not inhibited by the broad-spectrum caspase inhibitor zVAD-fmk or by expression of 9DN. We have also demonstrated that bcl-X<sub>S</sub> expression causes dissipation of the mitochondrial membrane potential (ΔΨ<sub>m</sub>). This loss of ΔΨ<sub>m</sub> was also not inhibited by zVAD-fmk or 9DN.

The mechanism by which the proapoptotic bcl-2 family members kill cells is still unclear, but a number of models has been proposed in the current literature including: (a) the direct release of cytochrome c from mitochondria and subsequent activation of effector caspases; (b) the inhibition of anti-apoptotic bcl-2 family members (by physical association); and (c) for BH3-only proteins, the liberation of pro-apoptotic bcl-2 family members that possess a BH1 and BH2 domain. These liberated proteins may then act through models (a) or (b), or through another mechanism, (d), the formation of new (or the alteration of existing) membrane-spanning channels.

The data presented here are primarily directed at testing the first of these models, activation of a caspase cascade initiated at the mitochondria. Our finding that caspase 9 is not required for cell death, rules out the reliance of bcl-X<sub>S</sub> on the cytochrome c-caspase 9-Apaf-1 apoptosome (18). In addition, zVAD-fmk (at concentrations up to 100 μM) was unable to inhibit bcl-X<sub>S</sub>-induced death in 3T3 cells. Although zVAD-fmk is not a potent inhibitor of some caspases (such as caspase 9), at 100 μM, it serves as an efficacious inhibitor of the presently described caspases (17). zVAD-fmk was, however, able to prevent Fas/Act D-induced cell death and is, therefore, functional in these cells at the concentrations used. To support the dominant negative caspase and caspase inhibitor data, fluorogenic caspase substrates were used to confirm the observation that caspases are not required for, nor activated during, bcl-X<sub>S</sub>-induced death in 3T3 cells. We used three different substrates to assay for the activities of the three caspase subfamilies and found no activation on expression of bcl-X<sub>S</sub>. We did however find significant caspase activity on treatment of the 3T3XS7.2 cells with Fas/Act D. These data argue strongly...
against model (a) as the mechanism for bcl-X₅-induced death in 3T3 cells. Nonetheless, our data do not exclude the possibility that there may be a caspase activity present in 3T3 cells that we have not inhibited with zVAD-fmk and that does not cleave any of the substrates we have used.

Interestingly, in the absence of detectable caspase 1-, 3-, or 9-like activities, we observed degradation of PARP after expression of bcl-X₅. This is not a unique phenomenon. Others have shown that PARP degradation occurs during apoptosis in the presence of zVAD-fmk (19) or during what the authors describe as necrotic cell death (also in the presence of zVAD-fmk; Ref. 20). In addition, on γ-irradiation-induced apoptosis of leukemia cells, Gong et al. (21) found that the serine protease inhibitors z-AAD-cmk and tosyl-lysine chloromethyl ketone (TLCK) prevented PARP cleavage in vivo. Casiano et al. (20) suggest that there are proteases activated in both apoptosis and necrosis but that there may be proteases unique to each type of cell death. We believe that a protease is being activated during bcl-X₅-mediated cell death that lacks caspase 1-, 3-, or 9-like substrate specificity, and that this unidentified protease is capable of degrading PARP.

Model b, the inhibition of anti-apoptotic bcl-2 family members, has not yet been evaluated in our system. bcl-X₅ has been shown to bind to bcl-X₇ and to abrogate its protective effect in other systems (7). However, the 3T3 cells used in this study express minimal levels of bcl-X₇; therefore, it seems unlikely that a primary action of bcl-X₅ is abrogation of bcl-X₇-mediated protection from death. These cells do contain significant levels of bcl-2, however, bcl-X₅ has not been shown to associate with bcl-2. This does not rule out the possibility of a bcl-X₇-like protein that can associate with bcl-X₅ and that has not yet been identified.

Another possibility was originally proposed by Muchmore et al. (22), who found structural homology between bcl-X₇ and the pore-
forming domains of certain bacterial toxins. It was hypothesized, and later shown, that bcl-X₆, bcl-2, and bax, can form conductive ion channels in lipid membranes and vesicles (3–6, 22). It has also been hypothesized, but not proven, that channel formation or alteration is one of the bcl-2 family’s mechanisms of action. This model is still highly theoretical because there is no in vivo evidence of channel formation to date. Bcl-X₆, however, is missing portions of the 5th and 6th α-helices, which are the putative pore-forming helices and, therefore, should not be able to form channels. This does not rule out the possibility that bcl-X₆ can, by BH3-mediated association, alter the structure and function of existing channels formed by other polypeptides or prevent the formation of new channels.

In conclusion, we have shown that bcl-X₆ induces acute cell death in 3T3 cells and that this death is both morphologically and biochemically different from Fas/CD95-induced death. bcl-X₆ expression does not require nor does it induce caspase activation during 3T3 cell death. Expression of bcl-X₆ does temporally coincide with loss of the ΔΨm and we hypothesize that this is the primary site of action for bcl-X₆. Whether this loss of ΔΨm requires opening of the permeability transition pore is still unclear. We have also shown that expression of a 9DN does nothing to prevent bcl-X₆-induced cell death in 3T3 cells. These findings suggest that bcl-X₆ kills 3T3 cells without caspase activity or activation. Therefore, bcl-X₆ may be able to induce cell death through a novel pathway for the pro-death bcl-2 family members.

ACKNOWLEDGMENTS

We thank Gabriel Nunez and Naohiro Inohara (University of Michigan, Ann Arbor, MI) for the Bax, Bak, and 9DN constructs and Todd S. C. Juan (Amgen Inc.) for the anticaspase 3 antibody. We also thank Alnawaz Rehemtulla for his guidance in this work.

REFERENCES

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Jordan S. Fridman, Mary A. Benedict and Jonathan Maybaum


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