Title
Structural and functional characterization of Bax induced apoptosis in the human fibrblast line GM701

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Publication Date
1999

Peer reviewed|Thesis/dissertation

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF BAX

INDUCED APOPTOSIS IN THE HUMAN FIBROBLAST LINE GM701

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL SCIENCES

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

To my parents, Dr. Fitch #1 and Dr. Fitch #2, for their never ending love and support. Their encouragement from the beginning made this work just a little bit easier, and having gone through it themselves, they knew why I could be so crazy, and were all the more supportive for it.

I especially dedicate this work to Greg, for his love and support (which is the kind that's a little more fun!). Knowing he would love a PhD, dentist, or whatever was sometimes all I needed.

ACKNOWLEDGEMENTS

I would first like to thank Tris Parslow for his support and encouragement, I will really miss my lovely window bay and the good times that were had in the lab. Chi-Ming Chang was my collaborator and my friend through much of this work, and I greatly appreciate all her help and support. I would also like to thank my chairperson, Caroline Damsky, for her guidance through my years of research, including my introduction into hard core cell biology. I would also like to thank Thea Tlsty for being a member of my thesis committee, and for all her generous teaching and sharing of her resources.

Lastly, I would also like to acknowledge some of the things that made graduate school fun. First, I made many lasting friendships through my interactions with students and faculty that I will always cherish. I want to especially mention Holly Strausbough and her husband Greg Hjelmstad for the many fun times we've had that made Friday worth working for. I also want to thank Brooke and Josh Greene for their years and years of friendship that I know will last through anything, including neglect due to thesis writing. I also have to laugh at the conversion of careers between myself and my twin, Karen, in finishing our degrees within months of each other, I guess genetic influence effects even graduate school. Great going Dr. Fitch #3! And lastly, as I have threatened to do all along, I have to thank the boys of summer, My San Francisco Giants, for having a great season and for making it so nice to watch a game after hours in front of a computer. I hope my success transfers to them so that we win a pennant! (You'll notice I didn't say world championship because I am now a trained skeptical scientist!)

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF BAX INDUCED APOPTOSIS IN THE HUMAN FIBROBLAST LINE GM701

By

Maureen E. Fitch

Abstract.

Bax is a proapoptotic member of the Bcl-2 family of proteins. It has been shown to induce apoptosis by associating with mitochondria and triggering cytochrome c release, which activates the caspase cascade. Bax can also kill some cells independently of caspases, but the requirements for such killing are poorly understood. Previous reports had shown that sequences in and around a conserved region in Bax, called the BH3 domain, are sufficient to confer killing activity when inserted into another protein. In this study, a series of point mutations were introduced into the BH3 domain of Bax in an effort to map residues necessary for killing; however none of these produced more than a partial loss of function. Similarly, replacing the BH3 region of Bax with that of the antiapoptotic protein Bcl-2 only partially reduced apoptogenic activity, implying that Bax exerts both BH3-dependent and BH3-independent effects that can lead to cell death. To analyze these effects further, we created a cell line in GM701 fibroblasts that inducibly expresses Bax. These cells underwent all the hallmarks of apoptosis when Bax expression was induced, and we determined that this cell death could be blocked completely by a broad-spectrum caspase antagonist. Even when caspases were inhibited, however, treating the Bax-expressing cells with oligomycin, a mitochondrial toxin, efficiently triggered apoptosis, as defined by characteristic surface phospholipid changes and condensation of the nucleus and cytoplasm. Bax and oligomycin thus synergize

to activate caspase-independent apoptosis in GM701 fibroblasts. We found that certain Bax BH3 mutants retained the ability to cause cytochrome c release and caspase mediated death, but could not support caspase-independent killing. Together, these findings illuminate a robust, caspase-independent pathway of apoptotic death that depends on the Bax BH3 domain and on effectors emanating from mitochondria.

Abstract Approved:  _____ Thesis Supervisor

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CHAPTER ONE

THE ROLE OF THE PROAPOPTOTIC PROTEIN BAX IN PROGRAMMED CELL DEATH

Programmed cell death was first described in the 1960's by pathologists who were looking at tissues, such as embryonic tissues and lymphatic structures, that were known to be hyperproliferative (1,3). Careful analysis of the number of cells produced through proliferation exposed a conundrum for tissue homeostasis: the number of cells produced did not equate with the size of the tissue. This led to the hypothesis that cells must also be removed in a manner that had yet to be observed, otherwise tissues would development rampant neoplasias. Cell death through necrosis had been studied for many years, and the histological details of cellular swelling and lysis, invasion by lymphoid cells, tissue destruction, and ultimately scarring, were well established. Necrotic cell death was not observed in these tissues unless an injury was inflicted, and so it had to be a different form of cell death. When proliferating tissues were examined for this non-inflammatory death, small numbers of dying cells were found with distinct morphological features. Careful study of whole tissue and cells in culture exposed a system of rapid cell death, condensation and engulfment by neighboring cells (2,5). By 1972, enough characterization had been done to warrant classification of this cell death, and so Kerr et al. ascribed the term apoptosis (from the Greek word for "dropping off" as in a petal from a flower) to this phenomenon (4). Among the hallmarks of apoptosis described then, and still used today, are plasma membrane blebbing, nuclear condensation

and breakdown, condensation of organelles, collapse of the cytoskeleton, and degradation of the cell into apoptotic bodies. These bodies are rapidly engulfed by neighboring cells, with no leakage of contents into the surrounding milieu; therefore no inflammatory response is generated.

Apoptosis has now been observed in every multicellular organism examined, as well as certain unicellular organisms (71, 78). Why a unicellular organism would commit suicide is not intuitively obvious, but it may be a mechanism to protect the whole colony in times of stress. Death of a single cell for the benefit of the whole organism is more easily understood and so most studies of apoptosis are done in metazoan cells. Apoptosis has been studied in many different stages of an organism's life. During embryogenesis, it can be observed during organogenesis, formation of limbs, and neuronal patterning. Apoptosis is also important for tissue homeostasis in the mature animal. Following cessation of lactation, mammary ducts involute through apoptotic cell death. The immune system uses apoptosis to remove autoreactive B and T cells, a process termed negative selection (49). Without this process, self-antigens would constantly be recognized, a destructive situation as evidenced by autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus. Another function of apoptosis in the immune system is killing of virally infected cells. Presentation of viral proteins by an infected cell through its MHC leads to recognition by T cells and a signal from the killer cell to the infected cell to apoptose. Just as was predicted by the initial studies, apoptosis prevents neoplasias from forming by removing damaged, misregulated, or simply unwanted cells. However, should a tumor arise, apoptosis can occur

spontaneously, or in response to chemotherapeutic agents, to cause the regression of the growth. Impairment of apoptotic responses can be found in many types of cancers, such as lymphomas, breast cancer, and neuroblastomas (7, 41, 26). On the other hand, too much apoptosis can also be harmful. Certain neural degenerative diseases, such as Parkinson's and Alzheimer's diseases, are the result of inappropriate neuronal apoptosis through mechanisms that are not well understood (120, 79).

Although apoptosis was described as early as 1966, understanding of its molecular basis was hampered by a lack of specific proteins to study. Through genetic analysis, Horvitz and his colleagues described three key *ced* (cell death) genes, which affected cell death in the nematode *Caenorhabditis elegans*. *C. elegans* was a perfect model organism to study because the fate of every cell could be followed. It had been shown that 1030 cells are formed through cell division of the fertilized egg, and yet precisely 131 cells are lost during development, so that the adult animal only contains 899 cells. It had also been determined that these 131 cells were lost through apoptosis. Hovitz used this knowledge to seek out mutations that would affect this carefully controlled cell death. They found approximately ten loci that affected cell death in various ways; most of these appeared to control processing of the "corpse", but three, *ced-3*, *-4* and *-9*, appeared to affect basic biological processes of apoptosis (24). Loss of function of the *ced-3* and *ced-4* genes resulted in abnormal cell survival, and so yielded adult worms with too many cells (10). Loss of *ced-9* resulted in the opposite phenotype, with more cells dying than normal. Genetic analysis indicated that *ced-9* acted upstream of *ced-3* and *ced-4*, suggesting that *ced-3* and *ced-4* activated

apoptosis, while *ced-9* inhibited this activity. Searching through the genetic databases, *ced-3* was found to be homologous to the mammalian cysteine protease interleukin 1-beta converting enzyme (ICE) and *ced-9* was homologous to the proto-oncogene *bcl-2* (16,10,11). At the time it was cloned, *ced-4* did not have any known homologues, but in 1997, Apaf-1 was cloned through a biochemical screen for apoptosis activating factors, and was found to be the elusive mammalian homologue of *ced-4*. (12,96)

The discovery that a cysteine protease was involved in apoptosis led to the search for more homologues. Over 10 mammalian homologues have been cloned to date, and the family has been given the name caspases (cysteine proteases that cleave after an **asp**artate residue)(48). Characterization of the caspases has led to a detailed understanding of their role in apoptosis (For review, see 92,123). Each protease is initially synthesized as an inactive zymogen. Activation of the enzyme occurs through proteolytic removal of a prodomain and cleavage into two active subunits. These subunits interact with another pair of subunits to form an active tetramer. Caspases have now been classified into subfamilies based on the length of their prodomains. The longer prodomains are believed to be important for targeting of the procaspases to certain structures as well as acting as regulatory mechanisms into which other apoptosis activating signals integrate into the caspase cascade. Intriguingly, the cleavage sites on the zymogen are consensus sites for caspases. This leads to the hypothesis that a stimulus that causes the cleavage of a small number of caspases can be amplified through a proteolytic cascade; the activated caspases then cleave critical cellular proteins, and ultimately cause a cell's death. Confirming

the idea that caspases are the executioners of apoptosis, overexpression of caspases in tissue culture systems can induce apoptosis, and the downstream events of this form of apoptosis are very similar to those induced by other inductive mechanisms, such as UV irradiation or chemical damage (16).

A key question in the study of apoptosis was how the proteolytic cascade was initiated. One mechanism, which was resolved early on, is that the serine protease Granzyme B can cleave certain caspases and cause their activation (90). Granzyme B is released by cytotoxic T cells, where it enters a cell targeted for death through a T cell derived pore, activates caspases, and starts the apoptotic cascade (53). However, other forms of apoptosis proceed normally in the presence of serine protease inhibitors, indicating there are mechanisms of zymogen cleavage that do not involve endogenous serine proteases. Studies in tissue culture cells showed that overexpression of caspases led to spontaneous zymogen cleavage and apoptosis (36,51,57), suggesting an innate mechanism of activation was present in the cell. Artificial creation of local high concentration of zymogens also led to caspase activation (113). These studies are the basis for the current hypothesis that physical clustering of zymogens allows a certain number of molecules to autocleave, and that this is the beginning of a proteolysis cascade. Confirmation of this hypothesis came with the understanding of how the *ced-4* gene product, and its recently cloned mammalian homologue Apaf-1, function. Apaf-1 is a 130kD protein that can serve as a docking site for the large prodomain of caspase-9 zymogen (82). Activation of Apaf-1 by an apoptotic signal causes Apaf-1 to multimerize, which clusters the bound caspase 9 zymogens and induces autocleavage (121).

A well-described caspase regulatory pathway is that of Fas activation of apoptosis (117). Fas ligand induces apoptosis by binding to the cell surface receptor, Fas, which causes a conformational change in that receptor which leads to the binding of FADD (33). FADD is an adaptor molecule that can interact with the large prodomain of procaspase 8 (previously known as FLICE or MACH)(62). Fas ligand is trimeric, and so interacts with three Fas molecules on the cell surface, causing several FADD and procaspase 8 molecules to cluster as well. The zymogens with smaller prodomains are believed to be the executioners of apoptosis as they show broader substrate specificity (68,130). They are activated by the regulatory caspases, and are responsible for most of the cleavage of downstream targets, but they too can feed into the caspase cascade by cleaving other caspases to augment the apoptotic response. The feedback loops within the caspase activation pathway probably function to ensure that apoptosis proceeds rapidly and efficiently.

Aside from other caspases, the list of downstream targets for the caspases has grown in recent years to include many regulatory and structural proteins. Most of the substrates that have been described are cleaved during apoptosis, but the consequences of this cleavage are not known. The best example of this type of substrate is poly(ADP-ribose) polymerase (PARP), which was historically one of the first substrates to be described as cleaved during apoptosis. Analysis using PARP deficient mice shows that loss of this protein has no effect on the induction or execution of apoptosis (75), and so the physiological significance of this substrate, if any, is not understood. PARP is involved in DNA repair and so loss of its function may prevent a cell from returning to normal if it somehow

escaped the apoptotic process. In contrast, certain caspase substrates become activated by cleavage and their role in apoptosis is clear. DNA fragmentation factor (DFF) is a nuclease, which was cloned by its ability to become activated by caspase cleavage, and it is responsible for much of the observed degradation of the chromosomes (6,83). Retinoblastoma protein (Rb) is an important tumor suppressor protein, which functions partly by inhibiting cell cycle progression, and loss of Rb augments apoptosis (37). Another type of substrate is that in which cleavage results in alteration in ability to form multimers (81). An example of this is the nuclear lamins, which lose their ability to form the complex interactions necessary to maintain the nuclear substratum once they have been cleaved (65).

More evidence that caspases play a pivotal role in apoptosis is the discovery of a number of viral proteins that specifically block caspases and thereby prevent viral induction of apoptosis. One of the first molecules described to have this function was the p35 protein from baculovirus (22). p35 can serve as a substrate for caspases, which cleave it at an aspartic acid residue, but the cleavage products then bind irreversibly to the enzyme active site and thereby inhibit further activity of the caspase (30,46). A protein from cowpox virus, Crm A, can function in a similar manner, and overexpression of either of these proteins can block activation of many types of caspases (123). Artificial inhibition of the caspases by addition of the synthetic caspase substrate, zVAD-fmk, can also function to block a wide array of apoptotic initiators. Another form of inhibitory protein is the family of iap (inhibitors of apoptosis) proteins. Iaps were initially cloned from baculovirus, but homologues have since been

identified in *Drosophila* and mammals (22,38,52). The mechanism by which these inhibitors function is not fully understood at this point, but they probably function to bind upstream regulators of the caspases and prevent their activation (54). One such example has been described in the tumor necrosis factor (TNF) pathway. TNF can induce proliferation or apoptosis depending on the state of the cell. The apoptotic pathway is initiated by a similar mechanism as the Fas system, so that binding of TNF by the receptor can causes oligomerization of caspase 9. A proliferative signal can also be generated through the TNF receptor by binding a different protein that eventually stimulates the cell through the NFκB pathway. Iaps bind to the TNF receptor and inhibit the formation of the caspase complex, thereby allowing the proliferative signaling complex to form (66). Further studies will surely expose many signaling pathways that decide between apoptosis and life by careful balance of these iaps and other caspase regulatory molecules.

In an effort to elucidate how caspases become activated, Wang and his coworkers made an exciting revelation about the involvement of the mitochondria in activating apoptosis. Mitochondria were known to undergo many changes during apoptosis, such as structural alterations, changes in the mitochondrial membrane potential ($\Delta\psi m$), and increased production of reactive oxygen species (ROS)(31). Indeed, some investigators had proposed that apoptosis occurred through malfunctioning mitochondria that generated ROS that would damage proteins, nucleic acids, and membranes, and therefore induce the cell to die (40). However, studies showing that cells continue to undergo apoptosis in the absence of respiring mitochondria (13), or under anoxic

conditions where few oxygen radicals are produced (44), had caused mitochondria to fall out of favor as a source of apoptotic signals. Wang and his colleagues took a biochemical approach to isolating apoptosis inducing factors by fractionating apoptotic cell extracts, adding them to non-apoptotic extracts, and looking for cleavage of a radiolabelled procaspase as an indicator of apoptosis activation. Using this *in vitro* system, they found several proteins that could activate apoptosis, one of which was the mitochondria protein cytochrome c (58). Cytochrome c (cyto c) is a 15kD soluble protein that resides in the mitochondria intermembrane space, where it shuttles electrons between complex II and III of the electron transport chain. Microinjection of cyto c into intact cells caused all of the hallmarks of apoptosis to occur, confirming its apoptosis activating activity. *In vivo* evidence demonstrates that cyto c is released from mitochondria soon after an apoptotic stimulus is given, and that blockage of this release can prevent many of the downstream events associated with apoptosis (80,94).

Another component that Wang et al. found to exert apoptosis inducing activity was Apaf-1 (96). As described above, Apaf-1 can physically interact with caspase 9 and cause its activation. Cyto c binds to Apaf-1, presumably inducing a conformational change that exposes the caspase-binding domain, and thereby initiates apoptosis through the activation of the caspase cascade (82). Another exciting result of the cloning of Apaf-1 was the realization that it contained sequences with similarity to Ced-4. Recently, another protein has been cloned which contains a Ced-4 homology domain, suggesting these proteins may also be part of a functionally redundant family. This protein, called FLASH, also binds a

regulatory caspase, and appears to function in a similar manner as Apaf-1 (128).

The realization that cyto c translocates out of the mitochondria in response to an apoptotic signal has focused a great deal of research on the question of how that translocation event occurs (86,101). The $\Delta\psi_m$ is established by the electron transport chain pumping protons out of the mitochondrial matrix. The primary function of this potential is to power the conversation of ADP to ATP by the F_1F_0ATPase, a large complex of proteins that form a channel in the inner mitochondrial membrane. Early during apoptosis, the $\Delta\psi_m$ is lost, but the mechanism by which this occurs is controversial. A permeability transition (PT) pore, or mega-channel, has been proposed as one way by which $\Delta\psi_m$ is lost and proteins, such as cyto c, escape. The PT has been shown to open early in apoptosis, but inhibition of it does not fully protect a cell from certain apoptotic stimuli, or prevent cyto c translocation (98,99), and so further study will be needed to determine how changes in $\Delta\psi$m are involved in apoptosis.

The genetic pathway described in nematodes also spurred the study of the *ced-9* mammalian homologue, Bcl-2. *bcl-2* was originally cloned as an oncogene that arose from the t(14:18) mutation found in a common human B cell lymphoma (7). Tissue culture studies of Bcl-2 indicated that its oncogenic activity stemmed not from conferring a proliferative advantage, but rather a survival advantage against death inducing signals (8). Bcl-2 has been shown to block apoptosis induced by a wide range of stimuli, including radiation, chemotherapeutic agents, hormones, removal from extracellular matrix, growth factor withdrawal, and negative selection in lymphoid cells (97). Bcl-2 has also been shown to functionally substitute for Ced-9 in the nematode, suggesting an

evolutionary conservation between key components of the apoptotic machinery (11).

As part of the study of Bcl-2 function, homologues were searched for and a large family has now been described, with members cloned from viruses and many different metazoan species. The homologous regions among this family can be broken up into four stretches of amino acids, now termed the Bcl-2 homology (BH) domains 1-4, with family members differing by which domains they contain. These regions are relatively small, comprising only 10 amino acids on average, but the degree of homology between members is very high. For example, the average homology over these four regions between Bcl-2 and another protective member, Bclxl, is 82%. Another homologue, Bax, has 3 of the 4 homology domains, within which it shares 67.5% homology with Bcl-2 (124). These domains function as protein-protein interaction sites, and are critical for homodimerization, as well as heterodimeric interactions among family members (28,72,89).

The Bcl-2 family of proteins has been challenging to study because it does not show any sequence similarity to known enzymatic motifs or characteristic binding domains. Because of this, early studies relied on stable overexpression in tissue culture systems, or loss of function through genetic disruption in mice. Although these techniques are not optimal, the initial data suggested that certain homologues, such as Bcl-2 and Bclxl, protected cells against an apoptotic stimulus, while others, such as Bax, enhanced an apoptotic stimulus. Genetic studies using transgenic *bcl-2* and *bclxl* mice have indicated that these two proteins function in very similar pathways to afford protection for cells in the

immune system (32). However, *bclxl* deficient mice die during embryogenesis (43), whereas *bcl-2* deficient mice are viable (17,19), suggesting that Bclxl may have broader protective functions outside of the immune system than Bcl-2. In contrast, stable, overexpression of Bax blocked Bcl-2's ability to prevent cell death induced by withdrawal of growth factors (18). *Bax* deficient mice exhibit less apoptosis in response to certain stimuli, also suggesting that Bax activates apoptosis (39). Like the *bcl-2* deficient mice, *bax* deficient mice are viable; suggesting there is functional redundancy for this activating activity. These initial findings established that the Bcl-2 family of proteins contains activators and inhibitors of apoptosis, and somehow integration of a death signal through these opposing effects leads to life or death.

A few years after the initial characterizations of Bax, a graduate student in our lab, John Hunter, was dissecting the critical domains for Bcl-2 and Bax function. He was using a transient overexpression system to express these proteins, and discovered that Bax was an apoptotic stimulus on its own when overexpressed in the human fibroblast cell line, GM701. The previous studies that had characterized Bax as an apoptotic enhancer had been done using stable overexpression of Bax in clonal cell lines. Creation of such lines masked the killing activity of Bax because massive death occurs through the selection process itself. Because he was using a rapid transient transfection system to analyze the apoptotic effects of Bax, John was able to appreciate this novel activity. Using domain-swapping mutagenesis, he was able to map this activity of Bax to 23 amino acids, a region that includes the BH3 domain. Further study showed that deletion of this region abrogated the ability of Bax to induce death when

overexpressed (55). Other labs have since described similar findings for several Bcl-2 homologues, including Bak, Bik, and Bid (34,35,29,67). Interestingly, Bik and Bid contain the BH3 homology domain only, confirming the importance of this domain for the induction of apoptosis. One confounding question of how this domain functions is the fact that this domain is present in several of the protective members as well (107).

The finding that certain homologues could induce apoptosis created a long standing controversy over which family members were the effectors of apoptosis and which acted as inhibitors of the effectors. For several years, it was postulated that Bcl-2 and other protectors were actively protecting the cell, and that proteins like Bax functioned by forming inactive heterodimers with these protective proteins (18). When it was discovered that Bax could induce apoptosis, the model flipped, so that it was hypothesized that Bcl-2 protected the cell by binding up Bax in inactive heterodimers (55). Support for either of these models was found in the fact that when mutations were made which interfered with dimerization activity, the pro- or anti-apoptotic effects of the protein were also destroyed (28,72,125). The problem with the single effector model was that it did not sufficiently explain why both Bcl-2-like and Bax-like proteins had effector functions, depending on the experiment, and so the controversy raged. To definitively address this question, mutations were made which created proteins that retained activity but could no longer heterodimerize with the antagonizing proteins. For example, a mutation in the BH1 domain of Bclxl was created so that this mutant no longer interacted with Bax or Bak by immunoprecipitation, but could still protect against sindbis virus induced

apoptosis (50). A similar example was described for Bax, in which substitution of certain BH3 residues resulted in loss of heterodimerization of Bcl-2 and Bclxl as assayed by yeast two-hybrid and mammalian cell immunoprecipitation, and yet these mutants could induce all of the characteristic effects of Bax induced apoptosis (63,74,95). Taken together, the results suggest that the ability to homo- or heterodimerize will influence the efficacy of each protein, but that each effector also exerts a dimerization-independent function. As a caveat to these findings, the physiologic importance of dimerization has recently been questioned by the realization that certain detergents and ion concentrations can affect the ability of these proteins to dimerize (77). Detergents are added during cell lysis in order to perform immunoprecipitations, and so may create artifactual dimerization conditions. The data from yeast two-hybrid studies also suggests a strong correlation between ability to interact and the ability to affect apoptosis, but yeast do not encode homologues to most of the apoptotic proteins, such as the Bcl-2 family or caspases, and so the relevance to mammalian apoptosis has been challenged (78). Regardless of whether or not the binding results are artifacts, all of the above experiments suggest that a more complex mechanism than simple competitive inhibition is responsible for the opposing functions of Bax and Bcl-2, and that both types of proteins may play an active role in the cell's surviving or dying (87).

The classification of the family members has been useful in establishing how complex the decision to apoptose is for a cell; however, it does not intuitively describe a mechanism by which these opposing functions may work. The nucleotide structure is unique, except among other family members, in that

there are no conserved enzyme motifs that would hint at a function. An exciting insight into a prospective function came with solving of the crystal structure of Bclxl (61). This protein folds into seven α-helices, several of which are made up of the BH domains. The striking resemblance of Bclxl to several bacterial toxins which form pores has led to the idea that Bcl-2 and other family members may function by forming pores. The structure of the pro-apoptotic protein Bid has also been solved, and although it only has the BH3 domain, it also shows a tremendous similarity to the pore proteins as well as Bclxl (126,129). The effector proteins localize to most membranes in the cell, including the nuclear envelope, endoplasmic reticulum, and mitochondria. These proteins may form a pore that changes the local chemistry at those membranes, and thereby regulates an apoptotic pathway that emanates from these sites. Supporting this idea, Bax and Bcl-2 have been shown to form ion channels with differing ion and voltage characteristics in synthetic lipid bilayers (85,88). Opposing channel functions would explain the antagonistic effects of these proteins, but more *in vivo* research is needed to verify that these proteins form pores that can affect apoptosis.

My research has focused on understanding the role of Bax in the induction of apoptosis. It had previously been established that Bax overexpression could induce apoptosis in many different types of cells, including fibroblasts, epithelial cells, and lymphoid cells. Bax had also been shown to inhibit apoptosis in a neuronal cell line (60), suggesting that cellular context can affect its function. Bax is widely expressed throughout development, and is also found in many adult tissues (25). It is present in tissues with characteristically high apoptotic rates, such as the germinal center of lymph nodes and the upper layers of gastric

glands, but it is also found in tissues that do not undergo high rates of apoptosis, such as neurons and fibroblasts. The presence of Bax in cells that are not actively undergoing apoptosis suggests that cells can inhibit its activity, and that one way an apoptotic signal acts is by releasing this inhibitory signal, and thereby allowing Bax to become active. One mechanism by which the cell may accomplish this is to compartmentalize Bax during normal growth where it cannot function, and to allow it to change compartments after an apoptotic signal is received. Support for this mechanism of Bax regulation can be seen in cell culture models, where Bax is normally found predominantly in the cytoplasm, however, after an apoptotic stimulus, it localizes primarily to membranes, including the mitochondrial membrane (76,93). Another mechanism by which apoptosis can be induced is through increasing the amount of Bax in the cell, and potentially titrating out an inhibitory signal (56). The *bax* promoter contains a binding element for the tumor suppressor protein, p53, and it has been shown that Bax RNA and protein levels increase after p53 activation (42). Loss of function of p53 is the most common mutation found in human cancer, and one of its important functions is to activate apoptosis in response to DNA damaging agents such as irradiation (9,84). Inducible expression systems have been created in the T cell line, Jurkat, to better define some of the distal events of Bax induced apoptosis (69,116). It was found in these cells that overexpression of Bax induced a classic appearance of apoptosis, such as caspases becoming active, the degradation of the cytoskeleton, cell shrinkage, and the nucleus degrading into small particles containing oligonucleosomal fragments. It was also observed that blockage of the caspase cascade by addition of zVAD-fmk could prevent several

of these events, however, the mitochondria lost their membrane potential, the nucleus showed signs of condensation and degradation, and the cells eventually died through a pathway which resembled necrotic cell death. These results suggest that Bax induced cell death occurs through both caspase activation and a separate pathway that is caspase independent.

My research has focused on extending the study of Bax induced apoptosis. Our lab had previously defined a region of 23 amino acids within Bax that was necessary for its apoptotic effect. This region corresponds to the BH3 region and my research initially focused on which amino acids within this were critical for function. I have determined that Bax can induce apoptosis in a BH3 independent manner, as mutation of this entire region resulted only in impaired induction of apoptosis. I have also established a fibroblast cell line that inducibly expresses Bax. This cell line was used to examine the downstream effects of Bax and further elucidate some of the mechanisms by which Bax exerts its proapoptotic effect. I have determined that our fibroblast line is resistant to Bax induced apoptosis if caspases are blocked, which is in contrast to the results found in Jurkat cells. I have examined several features of the mitochondria in Bax induced apoptotic cells, and found that several changes occur in the presence of caspase inhibitors. By addition of pharmacological inhibitors of the F_1F_0-ATPase, I have exposed a caspase independent mechanism of Bax induced apoptosis which is similar to that seen in the Jurkat cell line. I have further mapped this mitochondria specific component of caspase independent apoptosis to amino acids in the BH3 domain of Bax. Bax was also found to induce cyto c release and spatial relocalization of mitochondria, effects that are mediated through

separable regions from each other and from the F_1F_0-ATPase effect. My results indicate that Bax can induce apoptosis via multiple mechanisms, and they further illuminate the complex regulation of Bax induced apoptosis in mammalian cells.

CHAPTER TWO

DISSECTION OF THE STRUCTURAL REQUIREMENTS FOR BAX INDUCED APOPTOSIS

Bax is a 192 amino acid protein that is part of the expanding Bcl-2 family of apoptosis regulating proteins. Bcl-2 was originally cloned from the breakpoint t(14:18) found in a human follicular lymphoma (7). Bcl-2 and other protective proteins in the family, such as Bclxl, can protect metazoan cells from a broad range of apoptotic stimuli. The Bcl-2 homologues are defined by four regions, BH1-4, which can be found in varying combinations among the family members. Bax contains three out of four of the domains, lacking the BH4 domain that is found only in protective members. Bax was initially cloned by its interaction with Bcl-2, and its stable overexpression in lymphocytes was shown to inhibit Bcl-2's anti-apoptotic effects (18). A graduate student in our lab, John Hunter, studied Bax in a transient overexpression system in fibroblasts, and determined that Bax can induce apoptosis by itself (55). Transfection of the cells with the reporter protein β-galactosidase (β-gal) and Bax indicated that after 24 hours Bax could induce about 50% apoptosis in the transfected cells. This was a novel and exciting discovery because it was previously believed that Bax functioned solely by heterodimerizing with Bcl-2 and thereby interfering with Bcl-2's ability to prevent cell death.

At the time of this finding, it was not understood how any of the Bcl-2 family members affected cell death, and so John used mutational analysis to first

define where the killing domain of Bax was located. He constructed a series of chimeric proteins between Bax and Bcl-2, replacing the homologous regions of Bcl-2 with Bax residues, reasoning that he could convert Bcl-2 into a proapoptotic protein if the correct amino acids were swapped into Bcl-2. By constructing large swapping mutations, and then progressively smaller substitutions, John was able to isolate a region of 56 amino acids from Bax, which he termed C10, that induced a similar amount of apoptosis as was induced by full length Bax. He also found he could place this C10 region in a heterologous N-terminal location within Bcl-2, and that this chimera was able to induce 30% apoptosis as compared to 45% apoptosis when C10 was in the homologous position of Bcl-2. He also isolated a smaller chimera, termed C8, which contained only 23 amino acids of Bax (residues 55-77 of the mouse Bax protein) and which was able to induce 30% apoptosis when swapped into the homologous position of Bcl-2. Unlike C10, C8 was not capable of functioning when placed in a heterologous position in Bcl-2. This led to the hypothesis that the killing domain of Bax requires certain structural features which can be functionally substituted by Bcl-2, but that the majority of the killing activity could be isolated to the 23 amino acids of C8. Deletion of these 23 amino acids (residues 52 to 74) from Bax resulted in a protein with no apoptosis inducing activity. Several other groups have confirmed the presence of a killing domain in Bax, as well as defined similar killing domains in other family members (130). This domain includes the BH3 domain, a homology domain shared by many of the Bcl-2 family, including Bcl-2 and Bclxl. The killing domain that is the focus of my research is larger than the nine amino acid BH3 domain, which I will denote as BH3s, and so I will refer

to this region as BH3X, for BH3 extra.

The first portion of my thesis has focused on defining which amino acids within the BH3X are critical for apoptosis inducing activity. I first attempted to determine if there was a consensus site for the apoptosis inducing phenotype by constructing chimeric proteins similar to the Bcl-2/Bax C10 chimera using the Bcl-2 family homologues Mcl-1, Bak, and Bad. The second approach I used has been to make mutations within the killing domain of the full-length Bax protein, either through alanine substitution of specific amino acids, or through swapping mutations that replaced large portions of Bax with a heterologous protein. The results of both experimental approaches suggest that no single amino acid within the BH3X of Bax is necessary for the induction of apoptosis in a transient overexpression system. This suggests that Bax does not function solely by physical interaction with a downstream effector to induce apoptosis, and that there may be at least two killing domains within Bax that are capable of initiating an apoptotic pathway.

MATERIALS AND METHODS

Reagents

All chemicals, unless otherwise indicated, were purchased from Sigma. All restriction enzymes were purchased from New England Biolabs. The tetrapeptide inhibitors were purchased from Calbiochem. zVAD-fmk was purchased from Alexis. Oligonucleotide synthesis reagents were purchased

from Perseptive Biosystems.

Cell Lines

GM701 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamate (2 mM) (UCSF Cell Culture Facility) and 10% (v/v) fetal calf serum (Gemini BioProducts). All cells were maintained at 37°C in 5% CO_2 at 90% relative humidity.

Plasmid Construction and Mutagenesis

The Bcl-2 chimeras were made by isolating Mcl1 and Bad from a mouse cDNA library, and Bak from a human cDNA library using PCR. All the fragments had Bgl II at their 5' end, and Bam HI at the 3' end. They were cloned into the Bcl-2 Δ2 Bgl II site as described previously (55). The pSFFV-Bax/β–galactosidase expression plasmid was described previously (55). To created the HA tag at the N-terminus, the *bax* gene was subcloned into a pBluescript (pKS) vector by PCR. A HA gene fragment was cloned into the resulting Eco RI/Bgl II sites using the 5' oligo - AATTCATGTACCCTTACGACGTTCCTGACTACGCAGACGGGTC-CGGGGAGCAGA and the 3' oligo - GATCTCTGCTCCCCGGACCCGTC-TGCGTAGTCAGGAACGTCGTAAGGGTACATG. To kinase each oligo, 2μg of oligo was added to a reaction mix of 20μl with 1μl 10mM rATP, 1X kinase buffer, and 1μl of polynucleotide kinase. After the reaction was allowed to incubate at 37° for 1 hour, the reaction was terminated by a 20 minute incubation at 65°. The two oligos were mixed together, the proteins were removed by phenol chloroform treatment, followed by ethanol precipitation. The two oligos were then annealed at 37° for 30 minutes, and 5ng of the resulted annealed complex

was ligated overnight at 16° into the Eco RI/Bgl II sites in the N-terminus of *bax*. The epitope tagged *bax* was cloned back into the pSFFV-Bax/β–gal vector using an Xba I site in the pKS and an internal Pst I site in *bax*. To create some of the point mutants (See appendix), an internal Hind III site was introduced into basepairs 135-138 of *bax* , resulting in the missense mutation of threonine to serine at Bax residue 46. PCR mutagenesis was done by creating an oligo with the pertinent mutation, and running 30 cycles of PCR using the GeneMate® (ISC BioExpress). The resulting product was cut with enzyme, gel purified using Gene Clean® (Bio 101) and ligated overnight into the pSFFV-HABax/β–gal vector. Positve clones were screened by enzyme digest and confirmed by sequencing. To create Bax/ Bcl-2 and Bax/PARP chimeras, a similar kinasing and annealing procedure was used as described above. All plasmid DNA used for transient transfection were purified by cesium chloride density gradient.

Transient Transfection Studies

10μg of DNA was precipitated by addition of equal volume 25mM $CaCl_2$, and 2X HBS (1.5 mM sodium phosphate pH 6.5, .28M NaCl, 50nM Hepes pH7.5). The precipitate was added to GM701 cells which had been seeded the previous day on grided 60mM plates at a density of ~50000 cells. After 6-8 hours, the DNA was washed away with PBS, and fresh media was added. Unless otherwise indicated, the experiment was ended 18 hours later by removal of the media and fixation of the cells by addition of 1% gluteraldehyde/PBS. To visualize the transfected β–gal, its substrate 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside was added at a concentration of 2.5mg/ml in 3mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6$-$3H_2O$, 1 mM $MgCl_2$, 10mM KCl, .1% Triton X-100 made up in

.1M sodium phosphate buffer pH 7.5. After 3 hours at 37°, at least 200 blue cells were determined to be dead or alive based on morphology.

Western Blot Analysis

Cells were lysed in Triton-X lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1% [v/v] Triton X-100, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM Pefabloc [Boehringer Mannheim]). Aliquots containing 30-50 μg protein were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond filters (Amersham). Filters were pre-blocked for 15 min in Blotto (PBS with 3% [v/v] Carnation milk, 0. 1% [v/v] Tween-20), then incubated for 1 hr with primary antibodies in Blotto. Filters were then washed three times in PBS with 0.1% Tween-20 for 10 min each, incubated with secondary antisera in Blotto for 1 hr, washed again three times, and developed using ECL reagent (Pierce).

RESULTS

In an attempt to identify a consensus sequence for BH3X induced apoptosis, three chimeric proteins were generated that mimicked the chimeric protein Bcl2C10Δ2. Bcl2C10Δ2 was generated by cloning the C10 region of the mouse *bax* gene into an N-terminal deletion mutant of Bcl2 termed Δ2. It had previously been shown by our lab that placing the C10 region of Bax in a

heterologous position in Bcl-2 only lowered the apoptotic inducing ability of these 56 amino acids from 45% to 30%, suggesting that this domain contains most of the necessary information to induce apoptosis. We chose for study the BH3X regions of Bak, Bad, and Mcl-1. Bak and Bad had recently been cloned, and each had been shown to inhibit the protective effects of Bcl-2 when overexpressed in tissue culture cells (34,47). Mcl-1 had been described as a gene that is upregulated during human myeloid cell differentiation and its function was not known (15).

Using the consensus finding software, BestFit, which is based on a local homology algorithm designed by Smith and Waterman, I identified homologous amino acids in the various proteins that approximately corresponded to the C10 region of Bax (Figure 1A). Induction of apoptosis was analyzed by transient transfection of the chimeras into GM701 fibroblasts, along with the reporter plasmid encoding β-gal. The experiment was ended 24 hours after transfection by fixation of the cells, and the presence of the β–gal reporter protein was determined by addition of a substrate that is converted into an insoluble blue precipitate that accumulates in transfected cells. The percentage of apoptosis induced by each chimera was determined by counting the number of cells that had rounded up and appeared apoptotic, out of all blue cells. BakC10 clearly induced apoptosis at approximately the same rate as the BaxC10. BadC10 and Mcl-1C10 did not induce an appreciable amount of apoptosis over background (Figure 1B). Sequence alignment of each domain (Figure 2) indicates there is a high degree of homology among these proteins (conserved amino acids are indicated by bold lettering); however, there is not an obvious set of conserved

Figure 1. Comparison of BH3 killing activity among several Bcl-2 homologues. (**A**) Schematic representation of the chimeric proteins which were created for this study. Key residues are numbered above each construct. (**B**) Apoptogenic activities of each construct. GM701 fibroblasts were transiently transfected with the indicated plasmid along with a β-gal reporter plasmid, and apoptosis was scored 18 hrs post-transfection. Data shown are the mean ± standard deviation, n=3.

Figure 1

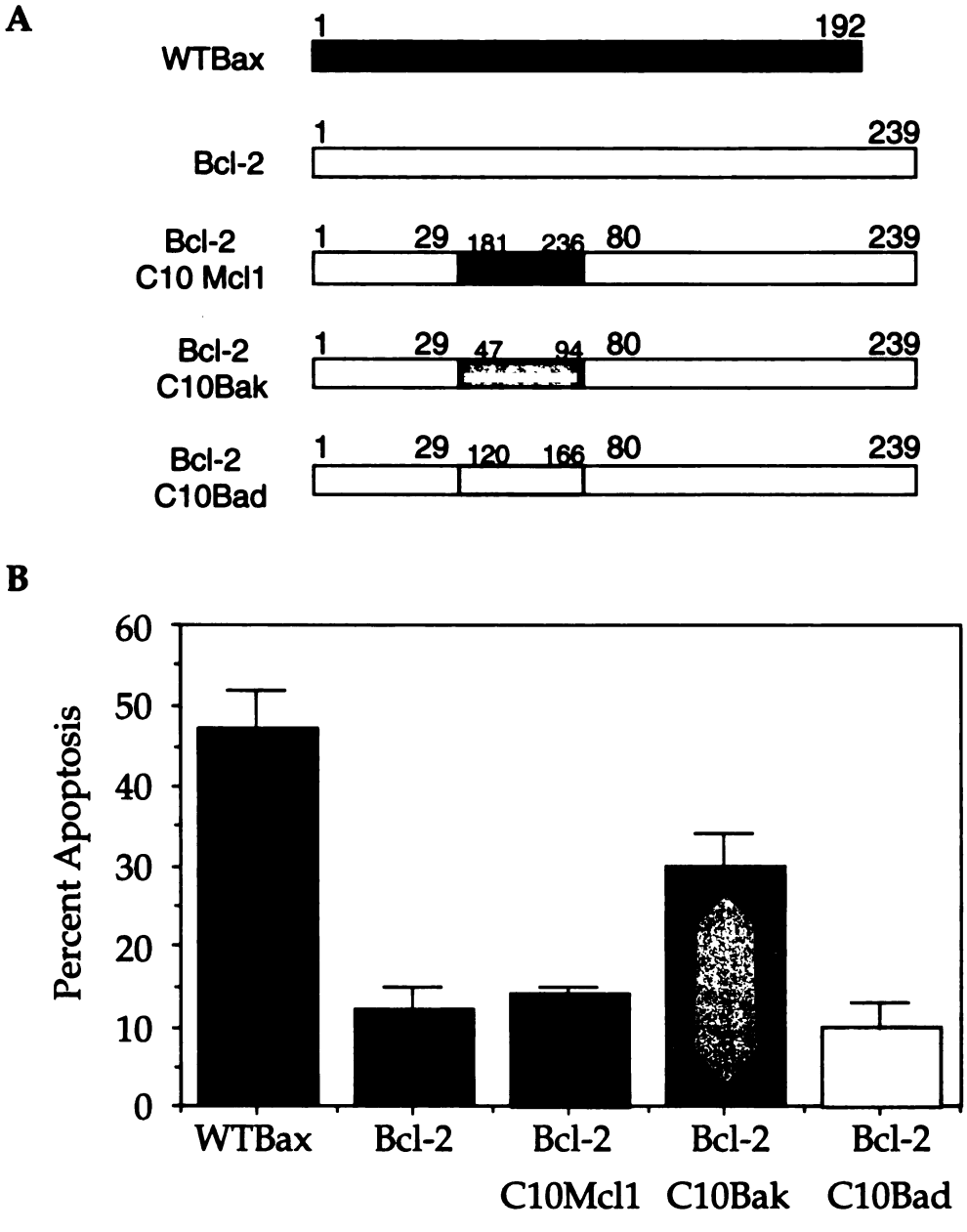
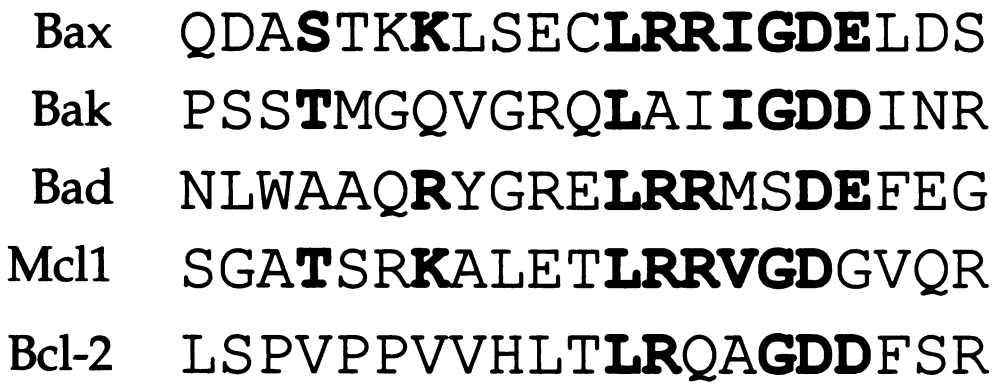

A
WTBax
1
192
Bcl-2
1
239
Bcl-2
C10 Mcl1
1
29
181
236
80
239
Bcl-2
C10Bak
1
29
47
94
80
239
Bcl-2
C10Bad
1
29
120
166
80
239
B
Percent Apoptosis
60
50
40
30
20
10
0
WTBax
Bcl-2
Bcl-2
C10Mcl1
Bcl-2
C10Bak
Bcl-2
C10Bad

Figure 2. BH3 domain alignment of the amino acid sequences of each of the homologues that were tested in Figure 1. Residues that are conserved among three out of the five homologues have been indicated by bold lettering.

Figure 2

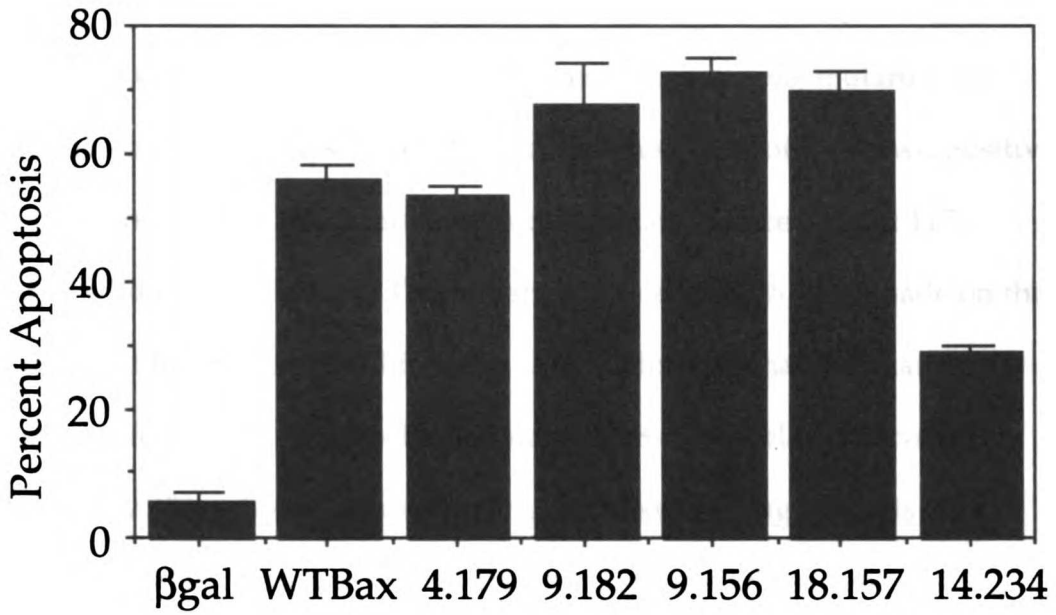

Bax QDASTKKLSECLRRIGDELDS
Bak PSSTMGQVGRQLAIIGDDINR
Bad NLWAAQRYGRELRRMSDEFEG
Mcl1 SGATSRKALETLRRVGDGVQR
Bcl-2 LSPVPPVVHLTLRQAGDDFSR

amino acids that are present in the death inducing homologues as compared to those that are not apoptogenic. From these results, I could not assess the structural requirements within the BH3X domain that are required for the induction of apoptosis.

To better define the required structural features for Bax-induced apoptosis, I used a systematic mutational approach, changing individual residues within the BH3X domain of the full length Bax protein, and assaying the ability of these mutants to induce apoptosis. The majority of the mutations converted two consecutive residues to alanines, however certain unique mutations were made to further define function. Apoptosis was again scored by the percentage of blue (β-gal positive) cells that displayed morphologic characteristics of apoptosis out of all blue cells. Representative experiments of six mutants are displayed in Figure 3. Wild type Bax induced between 50-60% apoptosis after 24 hours transfection. The β–gal control plasmid induced a background apoptosis of approximately 5%. Four out of six of the mutants induced approximately wild type killing levels. Mutant 14.234 induced only 25% apoptosis. This mutant has four alanines in place of the IGDE residues of the conserved BH3s region. In contrast to those results, the mutant 9.156 contains two alanines in place of the IG residues and induced about 20% more apoptosis than wild type. The complete results of the mutagenesis experiments have been summarized in Figure 4, where each mutant's apoptogenic activity is indicated as a percentage of wild type Bax induced apoptosis that was observed for each individual experiment. A complete list of each mutant with its original residues and the exact substitutions made can be found in the appendices. The majority

Figure 3. Representative experiment of mutagenesis of the BH3X domain of full length Bax. Using PCR mutagenesis, nucleotides which code for alanines were substituted into the wild type sequence of a mouse Bax gene. See Figure 4 for the description of each mutation. GM701 cells were transfected with the indicated plasmid along with a β-gal reporter plasmid, and apoptosis was scored 18 hrs post-transfection. Data shown are the mean ± standard deviation, n=3.

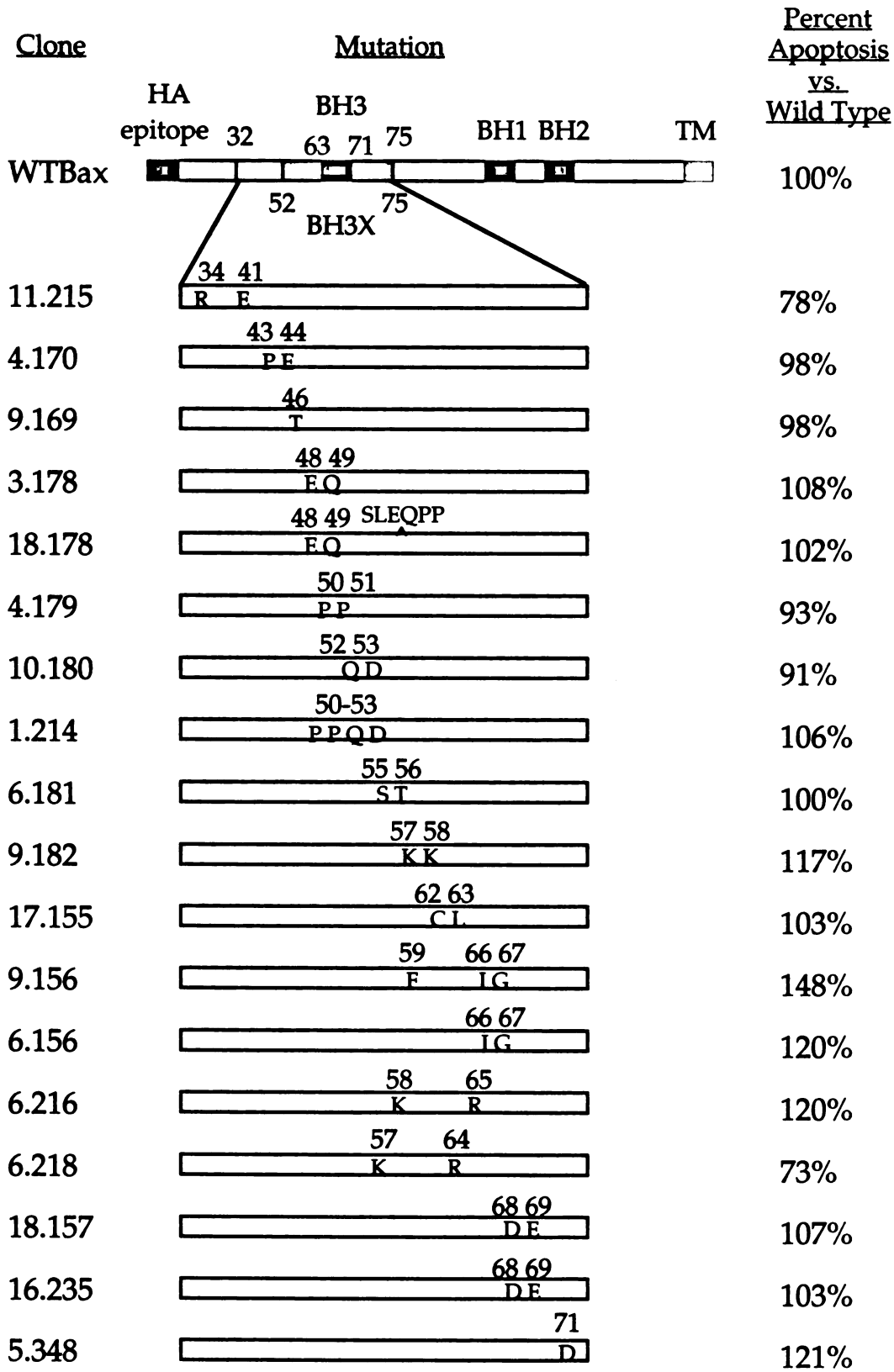
Figure 3


Percent Apoptosis
80
60
40
20
0
βgal
WTBax
4.179
9.182
9.156
18.157
14.234

of the mutations made in this region did not affect activity (e.g., 4.170, 6.181). Certain mutants appeared to induce slightly more apoptosis than wild type (e.g., 9.156 and 5.348). The BH3X region is composed of many charged residues that could function to make intra- or inter molecular ionic bonds, and so loss of these residues would presumably affect apoptogenic activity if such interactions were critical. I did not observe a significant loss of apoptosis when any charged residues were converted to uncharged alanines. For example, mutant 9.182 converts two consecutive lysine residues to alanines. Loss of these two positive charges did not seem to affect function as this mutant induced about 117% percent of wild type cell death. The mutants 6.216 and 6.218 were made on the prediction that this region could form an alpha helix, and that the charged residues may form a binding pocket. Mutant 6.218 did display a decrease of approximately 25% when compared to wild type, suggesting that this region may form secondary structure with partial function from these residues. Surprisingly, conversion of the most conserved residues of the BH3X region to alanines had little to no effect on Bax induced apoptosis. Mutants 17.155, 6.156, and 18.157 all contain mutations in one or more of the conserved residues which were isolated from the homologue consensus experiment, and each mutant induced apoptosis comparably to wild type. Mutant 16.235 coverts the negatively charged residues aspartate and glutamate at positions 68 and 69 of the BH3s region to positively charged arginines. This mutant also displays wild type killing, verifying a lack of specificity required for this region. However, when amino acids 66-69 were all converted to alanine, apoptosis was inhibited by over 40% (e.g.14.234), suggesting there is some degree of information found in this

Figure 4. Complete results of point mutagenesis of the BH3X domain of full length Bax. Substitution mutations were made by PCR mutagenesis as described in the Material and Methods. Each mutant is diagrammed in the figure by its position within Bax and the original amino acids which were changed (complete nucleotide sequence and amino acid substitutions can be found in the appendix). Each experiment included a WTBax and β-gal control, and was scored 18-20 hours post transfection. To account for the small amount of variation between experiments, each mutant is scored as a percentage of the WTBax induced apoptosis observed for each experiment.

Figure 4


Clone
Mutation
Percent Apoptosis vs. Wild Type
HA epitope
32
BH3
63 71 75
BH1 BH2
TM
WTBax
100%
52
75
BH3X
34 41
R E
11.215
78%
43 44
P E
4.170
98%
46
T
9.169
98%
48 49
E Q
3.178
108%
48 49 SLEQPP
E Q
18.178
102%
50 51
P P
4.179
93%
52 53
Q D
10.180
91%
50-53
P P Q D
1.214
106%
55 56
S T
6.181
100%
57 58
K K
9.182
117%
62 63
C L
17.155
103%
59
66 67
F
I G
9.156
148%
66 67
I G
6.156
120%
58
65
K
R
6.216
120%
57
64
K
R
6.218
73%
68 69
D E
18.157
107%
68 69
D E
16.235
103%
71
D
5.348
121%

region.

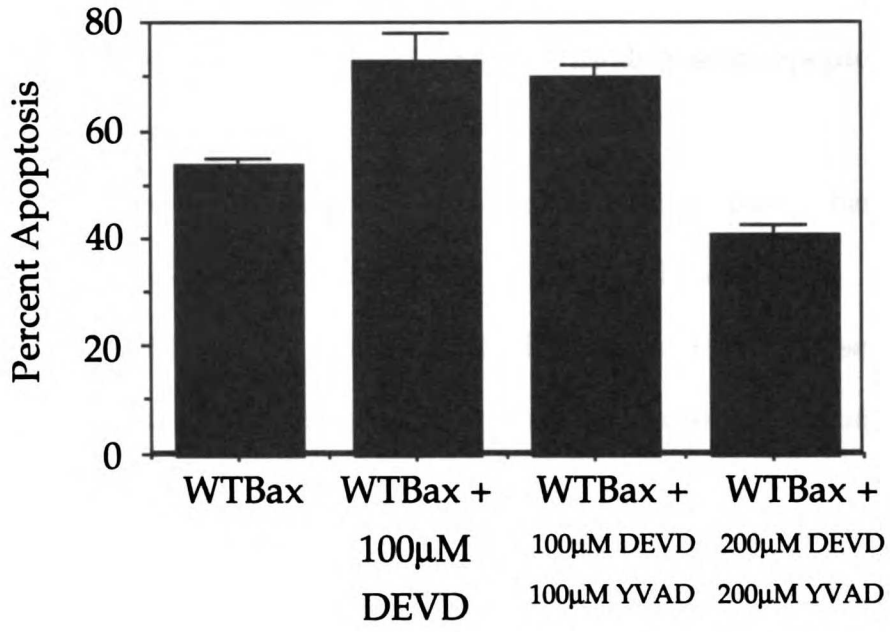
I next attempted to learn the role of the caspases in Bax induced apoptosis by adding specific inhibitors of this class of proteases. The caspases can be divided up into subgroups depending on their substrate specificity, and so inhibitors can be added to determine which subgroup is activated first in response to a particular apoptotic stimulus. I hypothesized that Bax could activate caspases by either of two mechanisms. It could directly activate one particular caspase, which would then activate the other caspases through a proteolytic cascade; or, Bax could activate a different apoptotic pathway that activated several different caspases at once. If the first hypothesis were true, it would be possible to block Bax induced apoptosis with a specific peptide inhibitor to one subgroup of caspases. If the second hypothesis were true, specific inhibitors would not block Bax induced apoptosis, and potentially complete blockage of caspase activation would not prevent Bax induced apoptosis.

The inhibitors I chose for study were the DEVD-CHO and YVAD-CHO peptides, which inhibit caspases 3 and 7 and caspase 1 and 4 respectively. I tested the ability of these peptides to inhibit wild type Bax induced apoptosis either individually, or in combination. I also tested the ability of the broad-spectrum inhibitor zVAD-fmk to block Bax induced apoptosis. zVAD-fmk had previously been shown to inhibit apoptosis induced by several apoptotic stimuli, including Fas ligation and viral infection. As shown in Figure 5, the specific inhibitors were relatively ineffective at inhibiting Bax induced apoptosis. Only when the two inhibitors were added in combination at the concentration of

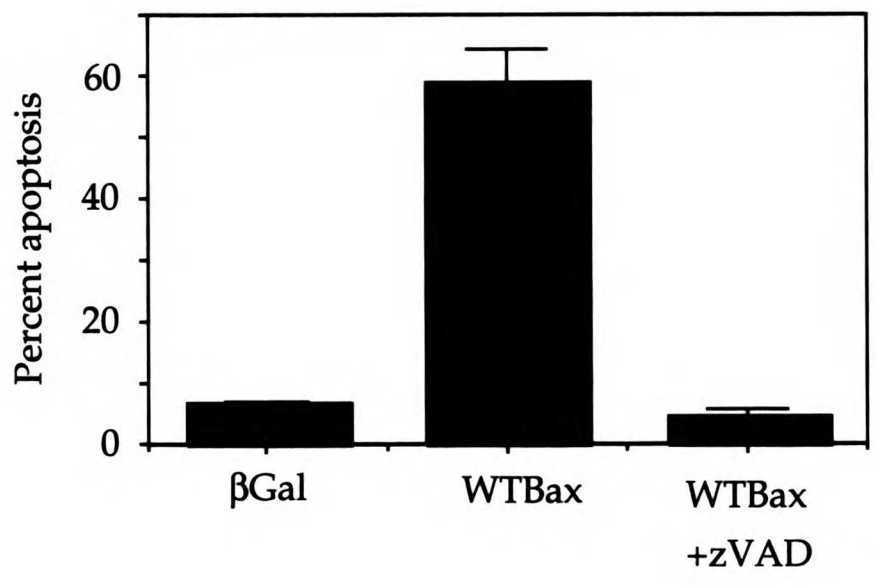
Figure 5. Analysis of Bax induced caspase activation, and their necessity for Bax induced apoptosis. (**A**) Inhibition of Bax induced apoptosis by specific tetra-peptide inhibitors of caspases. GM701 cells were transfected with WTBax as previously described. After washing away the transfection plasmids, peptide inhibitors were added at the concentrations indicated. Morphologic appearance of apoptosis was scored 18 hours later. (**B**) Same as (**A**), 40μM of zVAD-fmk was added to the media after the plasmids were washed away, and allowed to incubate 18 hours, at which time the induction of apoptosis was scored morphologically.

Figure 5

A


Percent Apoptosis
80
60
40
20
0
WTBax
WTBax + 100μM DEVD
WTBax + 100μM DEVD 100μM YVAD
WTBax + 200μM DEVD 200μM YVAD

B


Percent apoptosis
60
40
20
0
βGal
WTBax
WTBax +zVAD

200μM each did apoptosis drop from 50% in control cells to 40% in treated cells. This suggested that Bax activates several caspase subgroups during the apoptotic process. I next tested zVAD-fmk and found it to be a potent inhibitor of Bax induced apoptosis. The amount of apoptosis observed was less then the control β-Gal only, which I assumed was due to zVAD-fmk also inhibiting the apoptosis induced by the transfection procedure.

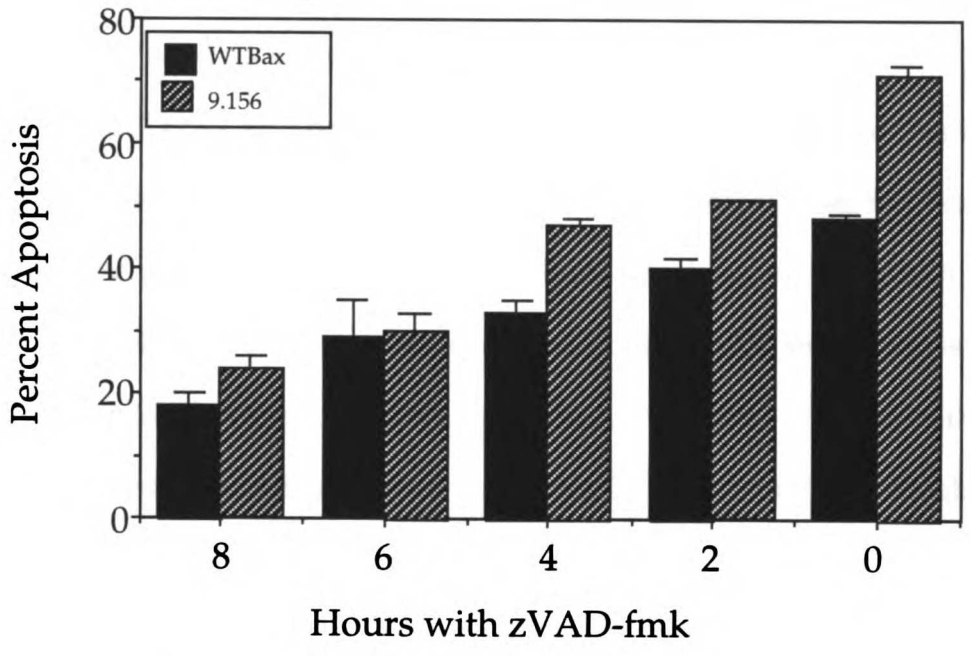
The ability of certain mutants to apparently induce more apoptosis than wild type was intriguing, and so I did further study on one of these "super-killers". I chose mutant 9.156 because it consistently displayed a 40% increase in apoptosis over wild type, and it was especially interesting because the isoleucine and glycine residues that are converted to alanines in this mutant are two of the amino acids that are highly conserved among several proapoptotic members. I reasoned that one mechanism by which this mutation could induce more apoptosis was if these residues were required for an interaction between Bax and a negative regulator. To examine this question, I took advantage of the apoptosis inhibiting effects of the pan caspase inhibitor, zVAD-fmk, to capture the apoptotic effects of each protein at discrete intervals. I first examined the rate of apoptosis induced by these proteins by addition of zVAD-fmk at various times after the start the experiment (Figure 6A). GM701 fibroblasts were transfected with either WTBax, or 9.156, for 24 hours as in the previous experiments. A time course of apoptotic activity was initiated at 16 hours post-transfection by the addition of zVAD-fmk at two-hour intervals to the media of separate plates. Similar to the previous studies, 9.156 displayed more apoptosis over WTBax at each interval examined. I reasoned that this effect might occur if one protein was

Figure 6. Analysis of killing of Bax mutant 9.156 verses WTBax over time.

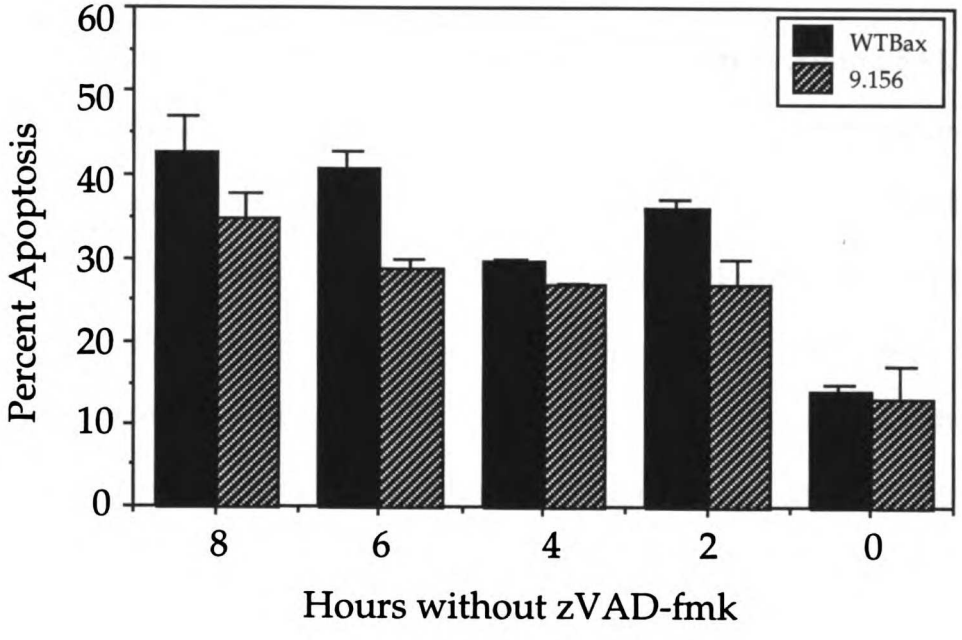
(**A**) GM701 cells were transfected with the indicated plasmids. Six hours later, the plates were washed to remove the plasmids. The media was replaced, and the experiment was stopped 18 hours post-transfection. During the experiment, apoptosis was allowed to proceed until it was stopped by the addition of 40μM zVAD-fmk at the indicated hours before the end of the experiment. (**B**) GM701 cells were transfected with the indicated plasmids and zVAD-fmk was included from time zero to prevent the procession of apoptosis. Six hours later, the cells were washed, and the media was replaced, as well as fresh zVAD-fmk was added. The experiment ended 24 hours later. To determine the rate of apoptosis of WTBax verses 9.156, the zVAD-fmk was removed from the media for the indicated hours prior to the end of the experiment, and apoptosis proceeded in those plates for the indicated time.

Figure 6

A


WTBax
9.156
Percent Apoptosis
80
60
40
20
0
8
6
4
2
0
Hours with zVAD-fmk

B


WTBax
9.156
Percent Apoptosis
60
50
40
30
20
10
0
8
6
4
2
0
Hours without zVAD-fmk

more stable than the other, and so to verify the super-apoptotic phenotype of 9.156, I designed the experiment to progress from the opposite direction (Figure 6B). I started the transfection with zVAD-fmk present at time zero, and then removed the zVAD-fmk at two hour intervals beginning at hour 24 and proceeding until hour 32. The caspase inhibition is reversible, and so this would allow apoptosis to proceed over the eight-hour experimental timeline. Examining the apoptotic characteristics of each protein from this direction exposed the opposite phenotype as from the previous experiments. 9.156 induced less apoptosis than WTBax at each interval examined, forcing the conclusion that 9.156 is impaired in inducing apoptosis. The results shown in Figure 6B also suggest that Bax-mediated caspase-induced death can proceed very rapidly, as apoptotic characteristics could be observed two hours after withdrawal of zVAD-fmk. To rule out stability differences, a western blot was done to verify protein expression of each mutant, and there does not appear to be a variance which would explain the different apoptotic effects for each protein (Figure 8A). I concluded that mutations made in Bax that impair its ability to fully activate the apoptotic process cause slower morphological manifestations of cell death such as rounding up and detaching. This leads to the apparent super-killer effect observed in the transfection assay for several of the mutants. In Chapter four, I show that a similar mutation to 9.156 is deficient in one aspect of Bax induced apoptosis, confirming the hypothesis that this mutation may cause apoptosis to proceed at a slower rate than wild type Bax induced apoptosis. Inhibition of the caspases by specific peptides also resulted in an apparent increase in apoptosis, suggesting that several caspase subgroups are responsible for the morphological

changes observed during Bax induced apoptosis. As more specific caspase inhibitors become available, it should be possible to demonstrate a difference in activation of individual caspases by wild type Bax verses 9.156 to further verify the effects of these proteins on activation of apoptosis.

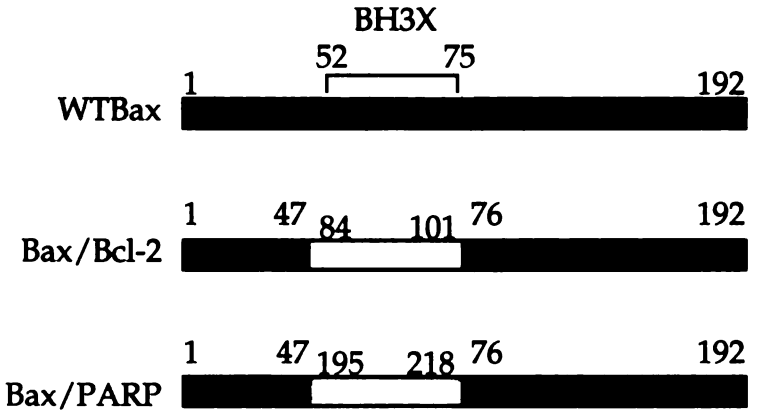
Complete mutagenesis of this BH3X region did not reveal any amino acids that were necessary for Bax induced apoptosis, and so I attempted to determine if there were any requirements within this domain by replacing the entire BH3X domain of Bax with homologous regions from two different proteins, Bcl-2 and PARP. I chose Bcl-2 because the previously swapping studies had successfully demonstrated the ability to define functional domains by constructing chimeras of these two proteins. I also chose to swap in a portion of the PARP protein because it is a well-defined caspase substrate that is cleaved very early in apoptosis. I was intrigued by a possible interaction between Bax and caspases because the sequence of amino acids 68-71 in Bax is homologous to several known caspase cleavage sites. These amino acids are in the conserved BH3 region, and putative caspase cleavage sites can be found in several other proapoptotic family members. Cleavage of Bax had not been observed during apoptosis, but I reasoned a cleavage site could also function as a caspase interaction domain. I constructed this chimera by selecting the correct number of amino acids to match the BH3X domain and thereby placed the caspase cleavage site of PARP in the homologous position of Bax.

The results of these swapping experiments are shown in Figure 7. Exchange of the BH3X between Bax and Bcl-2 resulted in only partial inhibition of the apoptosis inducing effect of Bax. Substitution of this domain with a

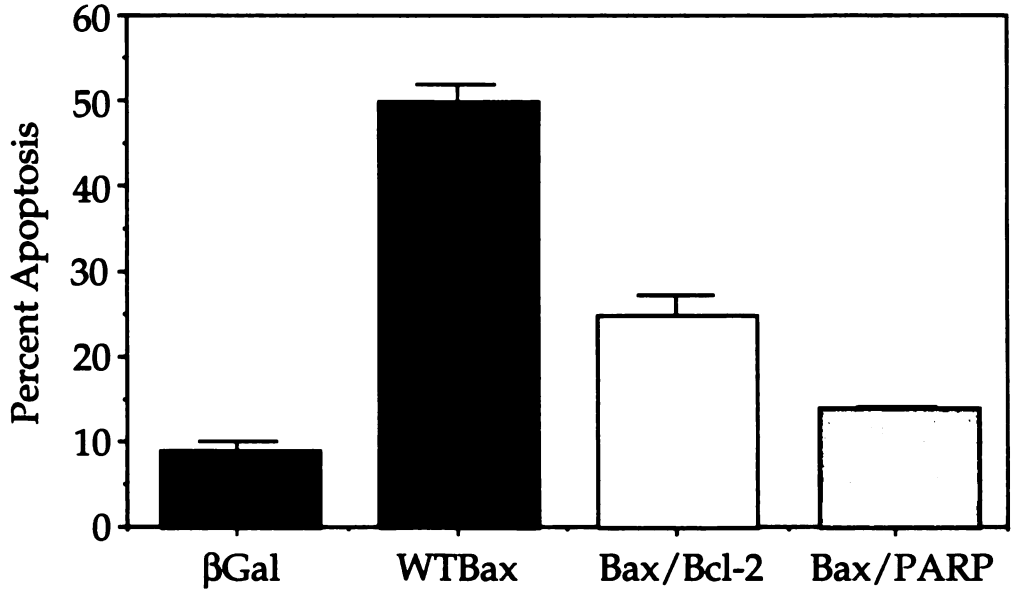
Figure 7. Determination of the structural requirements within the BH3X domain of Bax. (**A**) Swapping mutagenesis between Bax and Bcl-2 or PARP. The chimeric proteins were constructed by inserting the indicated amino acids of Bcl-2 or PARP into the BH3X domain of WTBax by oligonucleotide mutagenesis. The BH3X domain of Bax as defined earlier is indicated by brackets. (**B**) Apoptogenic activity of each construct. GM701 cells were transfected as described in Figure 3.

Figure 7

A


BH3X
52
75
1
192
WTBax
1
47
84
101
76
192
Bax/Bcl-2
1
47
195
218
76
192
Bax/PARP

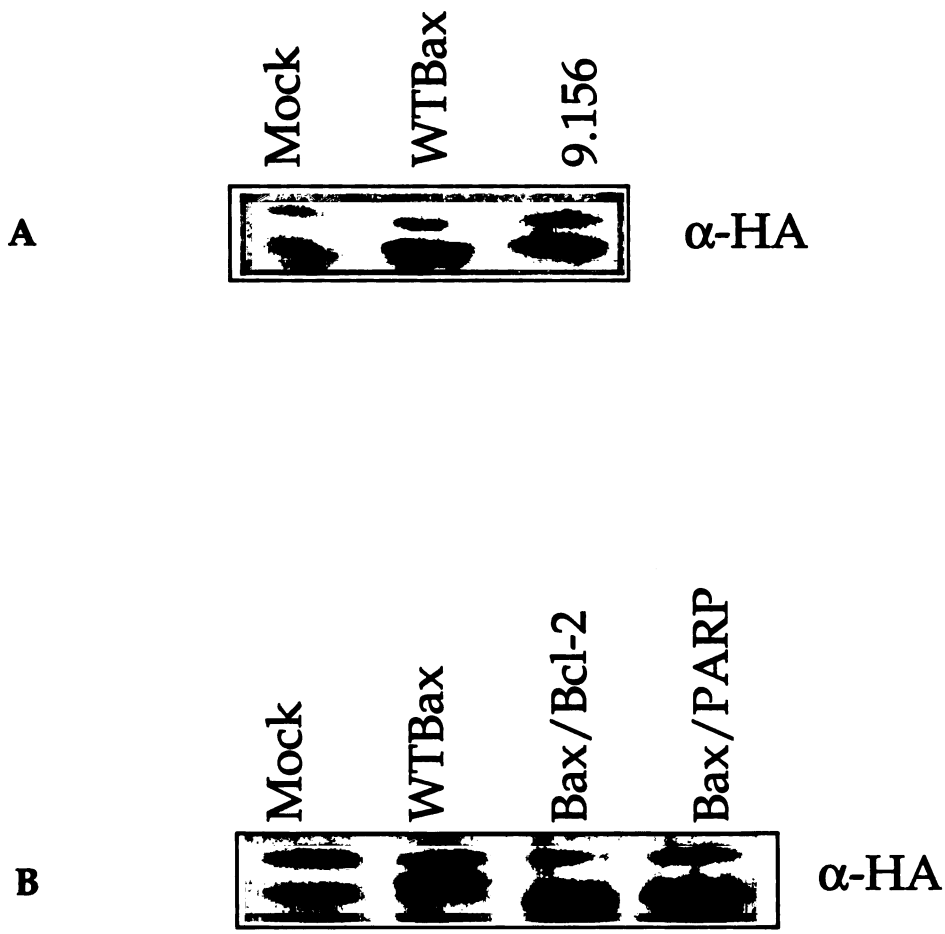
B


Percent Apoptosis
60
50
40
30
20
10
0
βGal
WTBax
Bax/Bcl-2
Bax/PARP

homologous region of PARP completely abolished the apoptotic inducing effects of Bax. Protein expression of the chimeras was confirmed by western analysis (Figure 8B). These results suggest that while particular amino acids in this region are not necessary for Bax to activate apoptosis, there is some information, possibly structural, which Bcl-2 can, and PARP cannot, provide. The latter result confirms earlier studies which showed that deletion of the BH3X region abolishes Bax function.

Figure 8. Western analysis of WTBax and mutants. (**A**) and (**B**) The indicated plasmids were transfected into GM701 cells, separated by SDS-PAGE, and probed with anti-HA.

Figure 8



CONCLUSIONS

I have completed a mutational analysis of a domain that is required for Bax induced apoptosis. This domain was previously described by its ability to transform the protective protein Bcl-2 into a proapoptotic protein. Deletion of this killing domain from Bax was shown to completely abrogate the apoptosis inducing phenotype of Bax. This suggested that all of the necessary information for Bax induced apoptosis could be dissected out of this region, which would then allow downstream effectors to be searched for that required these amino acids. In an effort to find these critical amino acids, I have used a transient transfection system in GM701 fibroblasts in which morphological features of apoptosis are used as the indicator of protein function.

I have determined that the BH3X region of Bax is not required for induction of apoptosis, as replacement of this entire region with the corresponding region from Bcl-2 resulted in only partial loss of function. These results confirmed our alanine mutagenesis studies, in which no mutation was identified which abrogated function by more than 40% as compared to wild type Bax. The complete loss of function of our PARP mutant suggests that there is some degree of information within this region that can be functionally replaced for by Bcl-2. These results are reminiscent of the C8 mutation that originally defined this death domain. C8 failed to induce apoptosis if it was placed in a heterologous position within Bcl-2, however the larger region, C10, could be placed in such a position. This indicates that Bcl-2 contains information that can functionally substitute for Bax to promote apoptosis. The determination of the three-dimensional structure of Bclxl and Bid family members offers an

explanation for this functional conservation. Bid is a proapoptotic member of the family with structural homology only in the BH3 domain; however, its three-dimensional structure is surprisingly similar to that of Bclxl. Intriguingly, the structure of these proteins closely resembles that of certain bacterial toxins that are able to form pores in membranes. Bcl-2 family members may therefore form opposing pores as one aspect of their functionality.

The determination that Bax can induce apoptosis without an intact BH3X domain suggests that Bax may activate apoptosis through multiple pathways. Clearly, the BH3X domain contains critical apoptosis inducing information because it can convert Bcl-2 into a proapoptotic protein. There must also be a second domain outside of this region that can also activate an apoptotic pathway. This domain has yet to be characterized, but the Bax/Bcl2 chimera protein described herein would be useful for finding this domain. I have characterized one aspect of these pathways through examination of the involvement of caspases in Bax induced apoptosis. I have found that the morphological features of apoptosis can be blocked for up to 32 hours by inhibition of the caspases with zVAD-fmk. Removal of this inhibition allows apoptosis to proceed and become discernable in less than two hours for both WTBax and a BH3X mutant, confirming the use of caspases by BH3X dependent and independent mechanisms.

Future studies need to be done to determine where the second apoptosis inducing region of Bax is located, as well as what structural features are required for its function. Proximal events of Bax activation have yet to be determined, and clearly this is of major importance to understanding how apoptosis is activated.

Once the elucidation of these events have been determined, these chimeric proteins will be valuable tools for the assignment of function to each domain within Bax.

CHAPTER THREE

CHARACTERIZATION OF AN INDUCIBLE SYSTEM OF BAX EXPRESSION AND APOPTOSIS IN GM701 FIBROBLASTS

Apoptosis is a complex process of cellular suicide that is tightly regulated in metazoan cells. In Chapter Two, I described how Bax contains at least two apoptosis inducing domains, but it was not known how these domains activated cell death. Several of the Bcl-2 homologues contain a transmembrane domain, and may function at membranes to regulate cell death, possibly through their ability to form channels with different ion preferences that then affect the chemistry of intracellular compartments, such as the mitochondria and the endoplasmic reticulum. Consistent with this hypothesis, it has recently become clear that mitochondria are important regulators of apoptosis. Mitochondria are the energy producing organelles of the cell, passing electrons through a complex transport system to create an electrochemical gradient, which they then use to convert ADP to ATP. Many apoptotic signals have been shown to induce the translocation of cyto c out of the intermembranous space into the cytosol (98,110,114), where it then interacts with the cytosolic protein, Apaf-1. This interaction that causes Apaf-1 to multimerize and thereby activate caspase-9 (121). Studies of mitochondria in apoptotic cells revealed that a "megachannel" opens and causes the dissipation of the electrochemical gradient, as well as spilling of mitochondrial contents into the cytoplasm. This channel is called the permeability transition (PT) pore and its regulation and composition are not well

understood (70).

In an effort to better understand the role of Bax in the induction of apoptosis, I have established a tetracycline repressible system of Bax expression in the human fibroblast cell line, GM701. I have isolated a clone, which I refer to as I-Bax, which undergoes many of the hallmarks of apoptosis upon removal of tetracycline (Tet) from the media. I have characterized the apoptotic process in I-Bax cells, and have determined that induction of Bax causes several changes to the mitochondria that occur through a caspase independent pathway. These caspase independent effects include the release of cyto c, perturbations to the $\Delta\psi_m$, and mitochondrial relocalization. However, Bax induced apoptosis requires caspase activation in GM701 cells, as blockage of these proteases with the pan caspase inhibitor zVAD-fmk completely inhibits the manifestations of apoptosis.

MATERIALS AND METHODS

Cell Lines

The human GM701 fibroblast line and all clones derived from it were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamate (2 mM) and 10% (v/v) fetal calf serum. All cells were maintained at 37°C in 5% CO_2 at 90% relative humidity. To establish Bax-inducible lines, GM701 cells were first transfected with pTET-Off regulatory plasmid (Clontech PT 3001-1), and a stable clone was selected according to the manufacturer's protocol. The pTRE-HABax response plasmid was constructed by inserting a murine *bax* cDNA with an N-terminal influenza hemagglutinin (HA) epitope tag (YPYDVPDYA) into the EcoRI-XbaI

sites of pTRE (Clontech). pTRE-HABax was transfected into the stable pTET-Off line along with a pTK-hygromycin selection plasmid. Stable transfectants were selected in 5 μg/ml tetracycline (Tet) and 0.2 mg/ml hygromycin, and then screened for cell death upon withdrawal of Tet. All Tet-dependent clones were supplemented with 5 μg/ml Tet every 1-2 days. To remove Tet, cells were washed in phosphate-buffered saline (PBS), trypsinized, and washed again in PBS before replating in fresh media.

Reagents

Rhodamine-conjugated anti-HA (12CA5) and FITC-conjugated goat anti-mouse sera were purchased from Boehringer Mannheim. Anti-human PARP (clone C2-10), anti-human Bclxl (B22630) and anti-cytochrome c (7H8.2C12) were purchased from Pharmingen. Anti-human Bcl-2 was purchased from DAKO (Clone 124). Anti-human actin (N350) was purchased from Amersham Pharmacia. Antibodies were used at dilutions recommended by the suppliers. Propidium iodide and saponin were from Sigma; tetracycline, hygromycin, and annexin V-FITC were from Calbiochem; zVAD-fmk was from Alexis Laboratories; $DiOC_6$, JC-1, and Mitotracker Green were from Molecular Probes. DAPI was purchased from Vector Laboratories.

Western Blot Analysis

Cells were lysed in Triton-X lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1% [v/v] Triton X-100, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM Pefabloc [Boehringer Mannheim]). Aliquots containing 30-50 μg protein were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond filters (Amersham). Filters were pre-blocked for 15 min in

Blotto (PBS with 3% [v/v] Carnation milk, 0. 1% [v/v] Tween-20), then incubated for 1 hr with primary antibodies in Blotto. Filters were then washed three times in PBS with 0.1% Tween-20 for 10 min each, incubated with secondary antisera in Blotto for 1 hr, washed again three times, and developed using ECL reagent (Pierce).

RNA Preparation and Northern Blot Analysis

RNA was isolated using the Trizol reagent (GibcoBRL). Aliquots (10 μg) were fractionated on formaldehyde/agarose gels, which were then soaked in water for 20 min and then soaked for an additional 15 minutes in 20X standard saline citrate (SSC, 1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). RNA was transferred overnight onto Hybond paper (Amersham) by capillary action, and crosslinked to it with ultraviolet light. Hybridization was done using radiolabelled DNA probes in ExpressHyb (Clontech) according to manufacturer's instructions, and detected by autoradiography. The HABax probe was a unique Kpn I/Bgl II fragment of pTRE-HABax. Glyceraldehyde 6-phosphate dehydrogenase (GAPDH) mRNA was detected with a Pst I/HindIII fragment of GAPDH cDNA.

Metabolic Labeling

Six hours before harvest, cells were changed into methionine-free DMEM containing 10% dialyzed fetal calf serum, 100 μCi/ml of ^{35}S-methionine (Amersham). Cells were harvested in Triton-X lysis buffer, and lysates were precleared overnight with 30 μl of ProteinA/G-Plus Agarose beads (Santa Cruz Biologicals). The following day, antibody was added and incubated for 3 hr at room temperature, then 30μl agarose beads were added for 3 hours. Beads were

washed three times in Triton-X lysis buffer, then eluted in 1X SDS sample buffer. Samples were fractionated on 14% PAGE, gels, which were then fixed, treated for 30 minutes with Amplify (Amersham), and analyzed by chemiluminescence.

Assessment of Apoptosis by Fluorescence Activated Cell Sorting (FACS)

Both floating and adherent cells were analyzed in all assays. For annexin V binding, cells were harvested using trypsin and then incubated with annexin V-FITC according to the manufacturer's instructions. For determination of DNA content, cells were harvested using trypsin, washed in PBS, pelleted, resuspended in 500 µl PBS, and then fixed by dropwise addition of 3 ml ice-cold 70% ethanol. After fixing for at least 30 minutes on ice, cells were washed in PBS, resuspended in PBS containing 20 µg/ml ribonuclease A and 20µg/ml propidium iodide, and then incubated for 15 min at room temperature before analysis. For analysis of mitochondrial function, either 50 nM $DiOC_6$ or 10 µg/ml JC-1 dye was added to the media, and cells were harvested 15 min later. All samples were analyzed using a Becton-Dickinson FACSort and Cellquest software, examining a minimum of 10,000 cells in each sample.

Subcellular Fractionation

Cells were grown as indicated. Floating cells were first collected, and adherent cells were harvested by trypsin treatment and added to the floating cells. After 2 washes in PBS, cells were subjected to isotonic lysis. Cells were incubated on ice for 15 minutes in isotonic buffer (200mM mannitol, 70mM sucrose, 1mM EDTA pH 8.0, 10mM Hepes pH 7.5), then disrupted by passage through a 23 gauge needle 15 times. Nuclei were removed by a 5 minute 500g spin, then membranes were separated from cytosol by a 1 hour 100,000 g spin, or a 20 minute 14,000

rpm spin.

Indirect Immunofluorescence

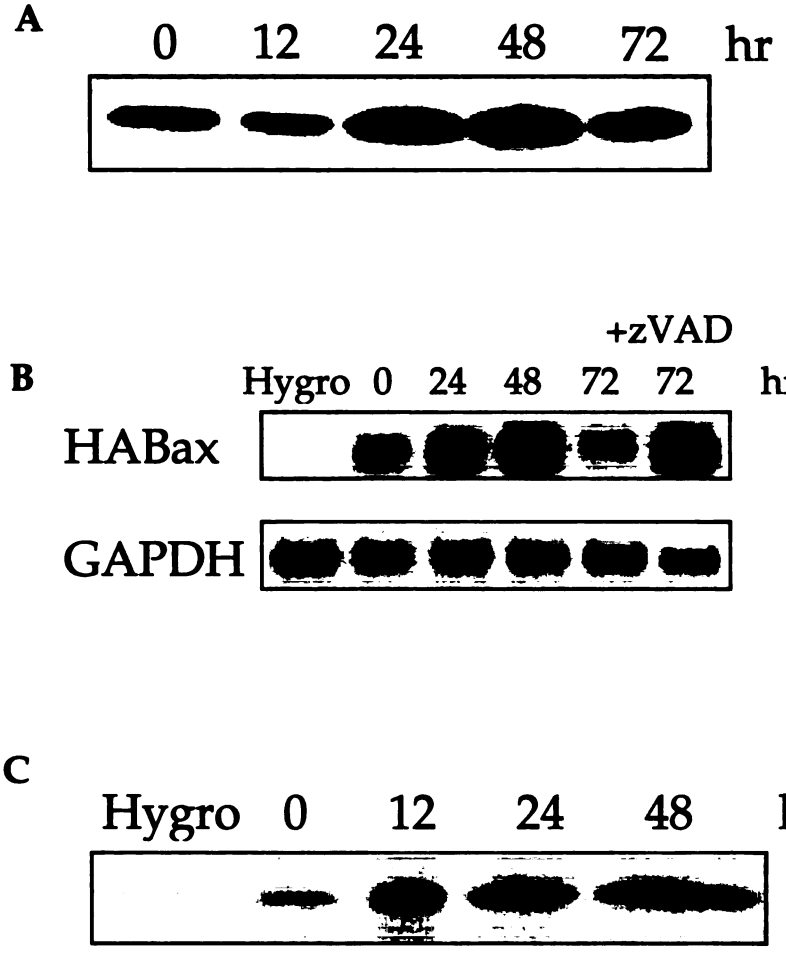
Cells grown on glass cover slips were transfected as described above, and fixed in 2% paraformaldehyde in PBS. Cells were washed in PBS with 0.03% saponin, then permeablized in PBS with 0.1% saponin and 1% BSA. Cells were double-labeled first with anti-cytochrome c at 1:75 dilution and FITC-conjugated goat anti-mouse IgG at 1:50 dilution, and then with rhodamine-conjugated anti-HA at 1:20 dilution. DAPI was added in the mounting medium (Vectashield, Vector Laboratories). Cells were examined using a Nikon Eclipse TE300 microscope and IP Lab Spectrum software (Scanalytics, Inc.).

RESULTS

To study the mechanisms of Bax-induced apoptosis, I transfected the human GM701 fibroblast cell line with an HA-epitope-tagged mouse Bax cDNA under the control of a tetracycline-repressible promoter. A gene cassette conferring hygromycin resistance was introduced simultaneously, and stably transfected colonies selected first for growth in hygromycin and tetracycline (Tet) were then screened to identify those that died following Tet withdrawal. One clone (which I designated I-Bax) was chosen for detailed study. As expected, withdrawing Tet from I-Bax cells caused increased expression of both the HA-tagged Bax protein and its mRNA within 24 hr, as judged from immunoblots (Fig. 9A) and Northern blots (Fig. 9B) that specifically detected products of the transgene. This induction was verified by HA-specific immunoprecipitation from biosynthetically radiolabelled cells, which revealed that synthesis of the tagged Bax protein increased markedly as early as 12 hr after drug withdrawal

Figure 9. HA-Bax protein and mRNA levels in I-Bax cells rise following withdrawal of Tet. Extracts were prepared either from Hygro control cells (Hygro), or from I-Bax cells at the indicated times following Tet-withdrawal. (**A**) Induction of HABax protein. Whole-cell lysates prepared from Bax cells were fractionated by PAGE and immunoblotted using an HA-specific antibody. (**B**) Northern blot analysis of HABax mRNA induction. RNA harvested from cell lysates was probed for sequences at the unique 5′ end of the transgene (HAbax) or for endogenous GAPDH sequences (GAPDH). For the "+zVAD 72 hrs" sample, zVAD-fmk was added to I-Bax cells at the time of Tet-withdrawal and then was replenished daily. (**C**) New synthesis of HABax protein. Hygro or I-Bax cells were incubated with ^{35}S-methionine for 6 hr prior to harvest; cell lysates were then immunoprecipitated with HA-specific antibody, and the precipitates were fractionated by PAGE and analyzed by autoradiography.

Figure 9


A
0 12 24 48 72 hr
+zVAD
B
Hygro 0 24 48 72 72 hr
HABax
GAPDH
C
Hygro 0 12 24 48 hr

(Fig. 9C). Even before withdrawal, levels of the tagged protein and mRNA were higher in I-Bax than in GM701 cells that had been transfected with the hygromycin-resistance marker only (hygro), implying that Tet repression of the Bax gene was incomplete.

Signs of apoptosis became apparent in I-Bax cells soon after Tet-withdrawal. I first tested for changes to the plasma membrane which are known to occur early in apoptosis by examining the binding affinity of annexin V and exclusion of the DNA dye propidium iodide by unfixed I-Bax cells. Annexin V is a phospholipid binding protein with high affinity for phosphotidylserine, a lipid that is normally found only on the inner leaflet of the plasma membrane. Exposure of this lipid on the outer plasma membrane during apoptosis serves to induce engulfment by neighboring phagocytic cells and can be detected by addition of annexin V conjugated to fluorescein isothiocyanate (FITC) (45). Propidium iodide is a fluorescent DNA-intercalating dye that cannot penetrate membranes and so distinguishes between apoptotic cells, which have an intact membrane until very late in apoptosis, and necrotic cells, which do not. After 24 hours without Tet, I-Bax cells showed an increase in annexin V binding by FACS analysis, without showing a similar increase in propidium iodide staining, demonstrating that these cells are dying through apoptosis and not necrosis (Fig. 10A). Bax induced apoptosis increased over time to affect 20-25% of all cells within 72 hr (Figure 10B) as measured by this assay. I also tested if mitochondria were affected by Bax induction. The lipophilic fluorescent dye $DiOC_6$ is specifically concentrated into the matrix of mitochondria that have an intact mitochondrial transmembrane potential ($\Delta\psi_m$) (31). The opening of the PT pore

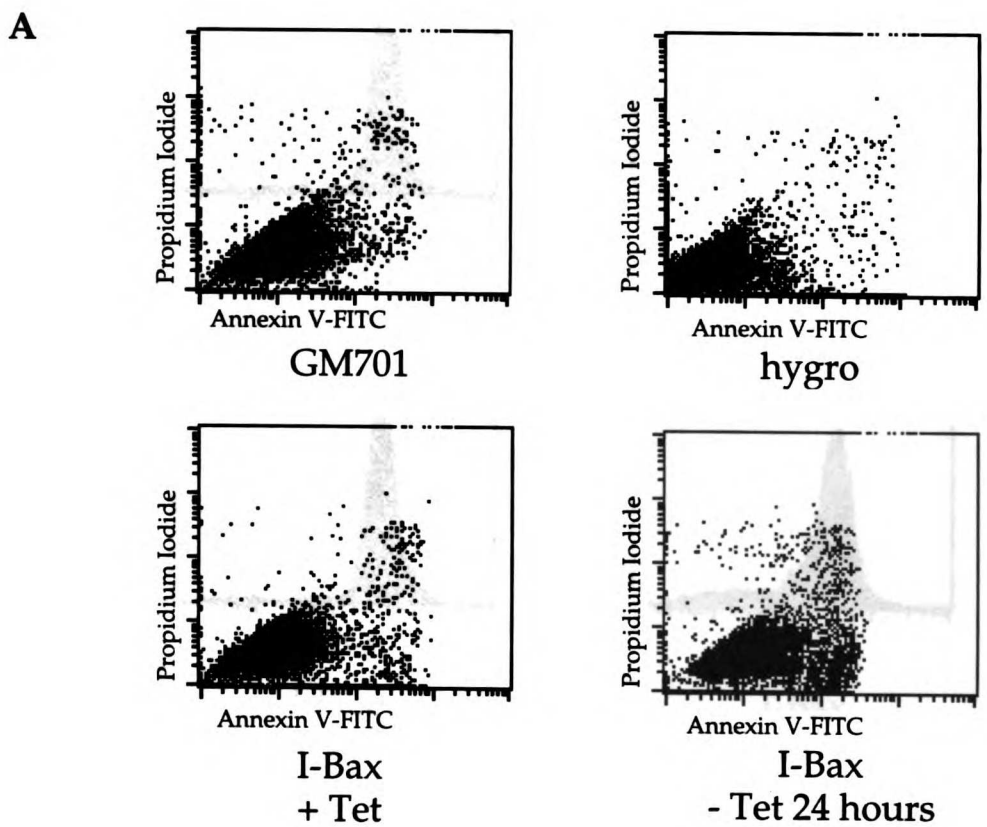
Figure 10. Withdrawal of Tet induces apoptosis in I-Bax cells. I-Bax cells were analyzed after growth for indicated times with or without Tet.

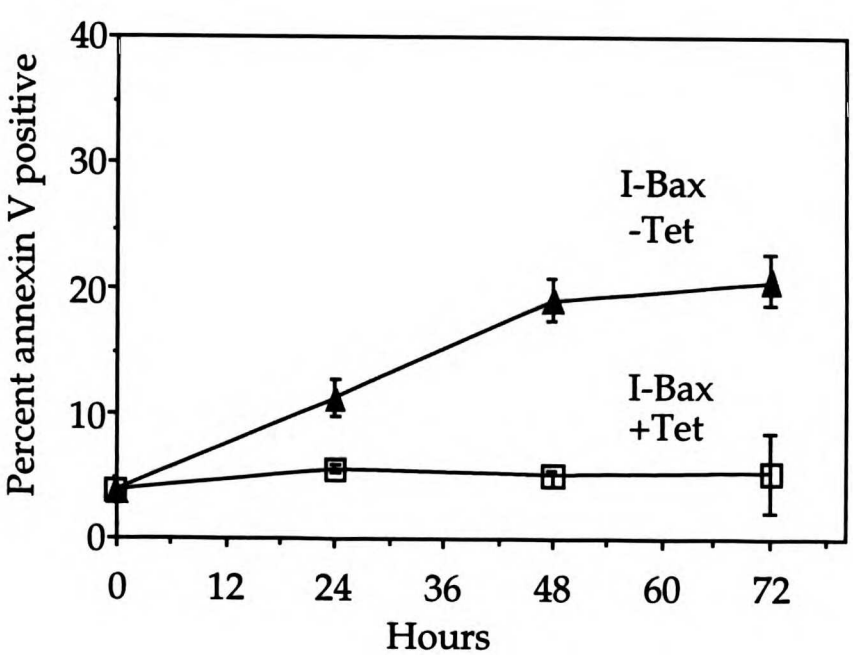
(**A**) Representative FACS analysis of cells with exposed surface phosphatidylserine using annexin V-FITC and propidium iodide (x =FL-1, y=FL-2). Cells were harvested after 24 hours, hygro and I-Bax -Tet cells had been grown without Tet during the previous 24 hours.

(**B**) Quantification of annexin V-FITC binding over 72 hours. n=3.

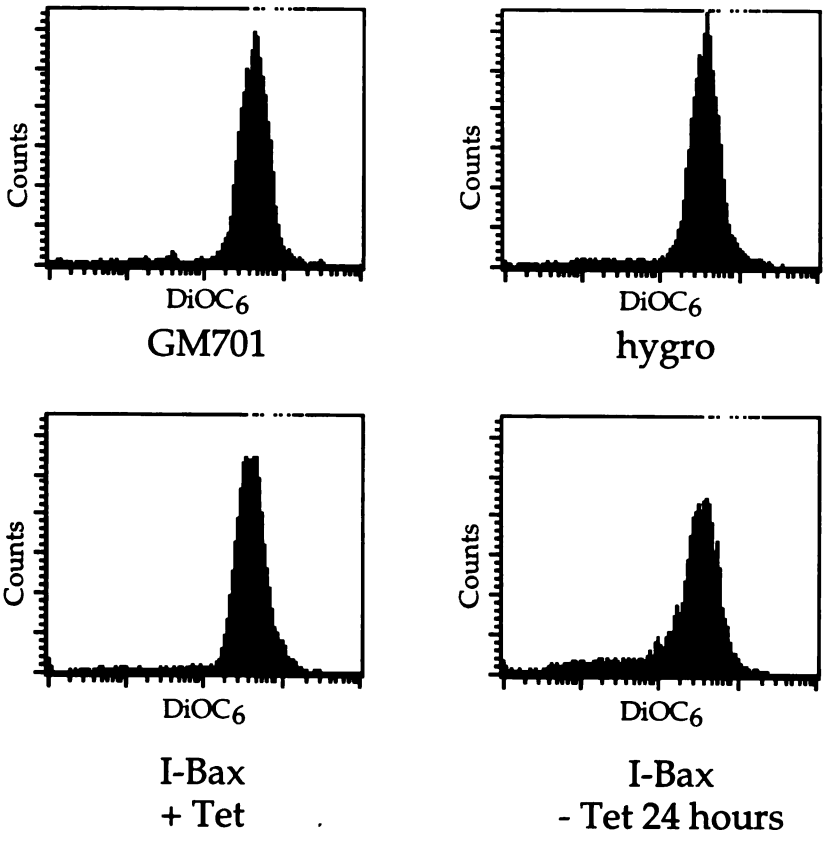
(**C**) Representative FACS analysis of cells exhibiting a reduced $\Delta\psi_m$ as assayed by the dye $DiOC_6$ (x=FL-1). (**D**) Quantification of loss of $\Delta\psi_m$ over 72 hours. n=3. (**E**) Cleavage of endogenous PARP protein, a caspase substrate. Cell lysates were prepared at the indicated times after Tet-withdrawal, 50μg of each sample was fractionated by PAGE, and immunoblotted using mouse anti-PARP antibody. Arrows denote the 115-kD full-length PARP (upper) and 85-kD cleavage product (lower). (**F**) Growth kinetics were assayed by visually counting viable (trypan blue-negative) cell density in duplicate experiments, expressed as a multiple of the initial plating density. Results are the mean of two experiments $\pm$ standard deviation.

Figure 10

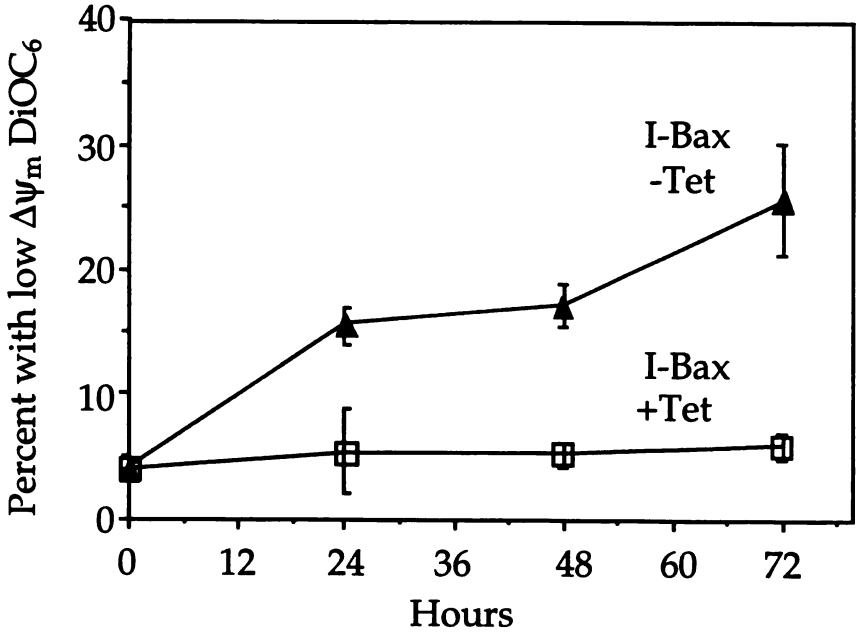

A
Propidium Iodide
Annexin V-FITC
GM701
Propidium Iodide
Annexin V-FITC
hygro
Propidium Iodide
Annexin V-FITC
I-Bax
+ Tet
Propidium Iodide
Annexin V-FITC
I-Bax
- Tet 24 hours


B
Percent annexin V positive
40
30
20
10
0
I-Bax
-Tet
I-Bax
+Tet
0
12
24
36
48
60
72
Hours

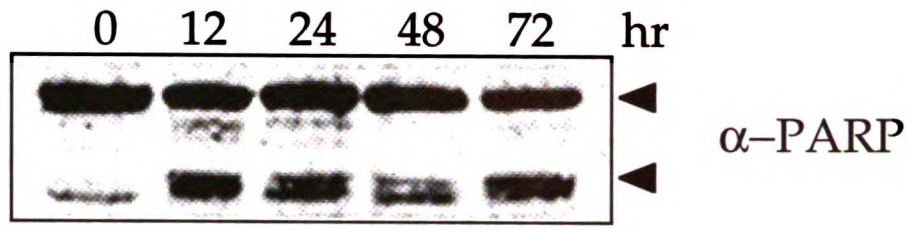
C


Counts
DiOC6
GM701
Counts
DiOC6
hygro
Counts
DiOC6
I-Bax
+ Tet
Counts
DiOC6
I-Bax
- Tet 24 hours

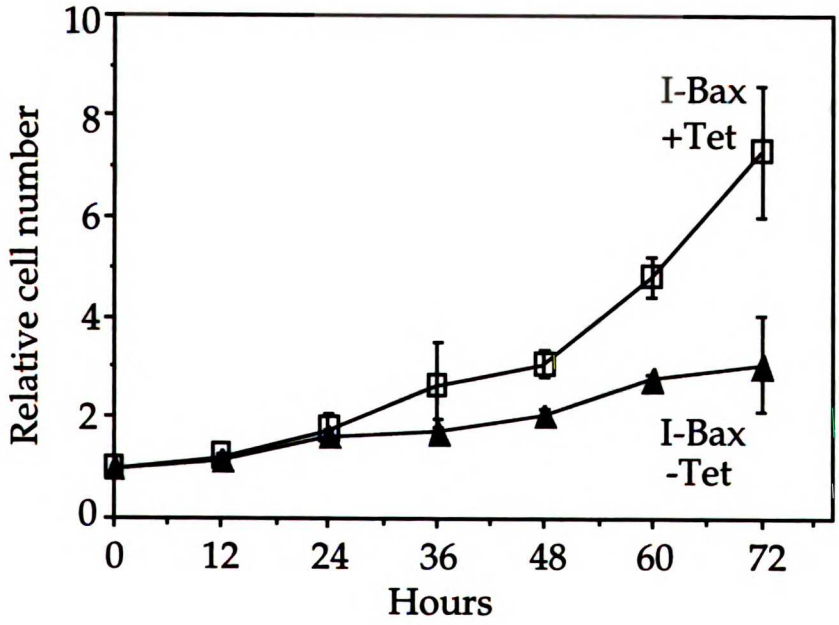
D


Percent with low Δψm DiOC6
40
30
20
10
0
I-Bax
-Tet
I-Bax
+Tet
0
12
24
36
48
60
72
Hours

E


0
12
24
48
72
hr
α–PARP

F


Relative cell number
10
8
6
4
2
0
0
12
24
36
48
60
72
Hours
I-Bax
+Tet
I-Bax
-Tet

early during apoptosis causes the dissipation of $\Delta\psi_m$ and therefore apoptotic cells bind less of this dye than live cells. Similar to the annexin V data shown above, upon removal of Tet, I-Bax cells showed signs of apoptosis within 24 hours, as demonstrated by the reduced binding of $DiOC_6$ (Figure 10C), and 25% of all cells showed a reduction in $\Delta\psi_{m\,by}$ 72 hours (Figure 10D). Comparable percentages of cells also became stainable with trypan blue at these times (data not shown). Caspase activation could be detected as early as 12 hr post-withdrawal, as evidenced by cleavage of endogenous PARP protein (Fig. 10E). The cumulative effect of these ongoing deaths was also evident in the growth kinetics of the population, in that net proliferation of the cells was reduced by more than 70% after 3 days (Fig. 10F) and no viable cells remained after 5 days without Tet (data not shown).

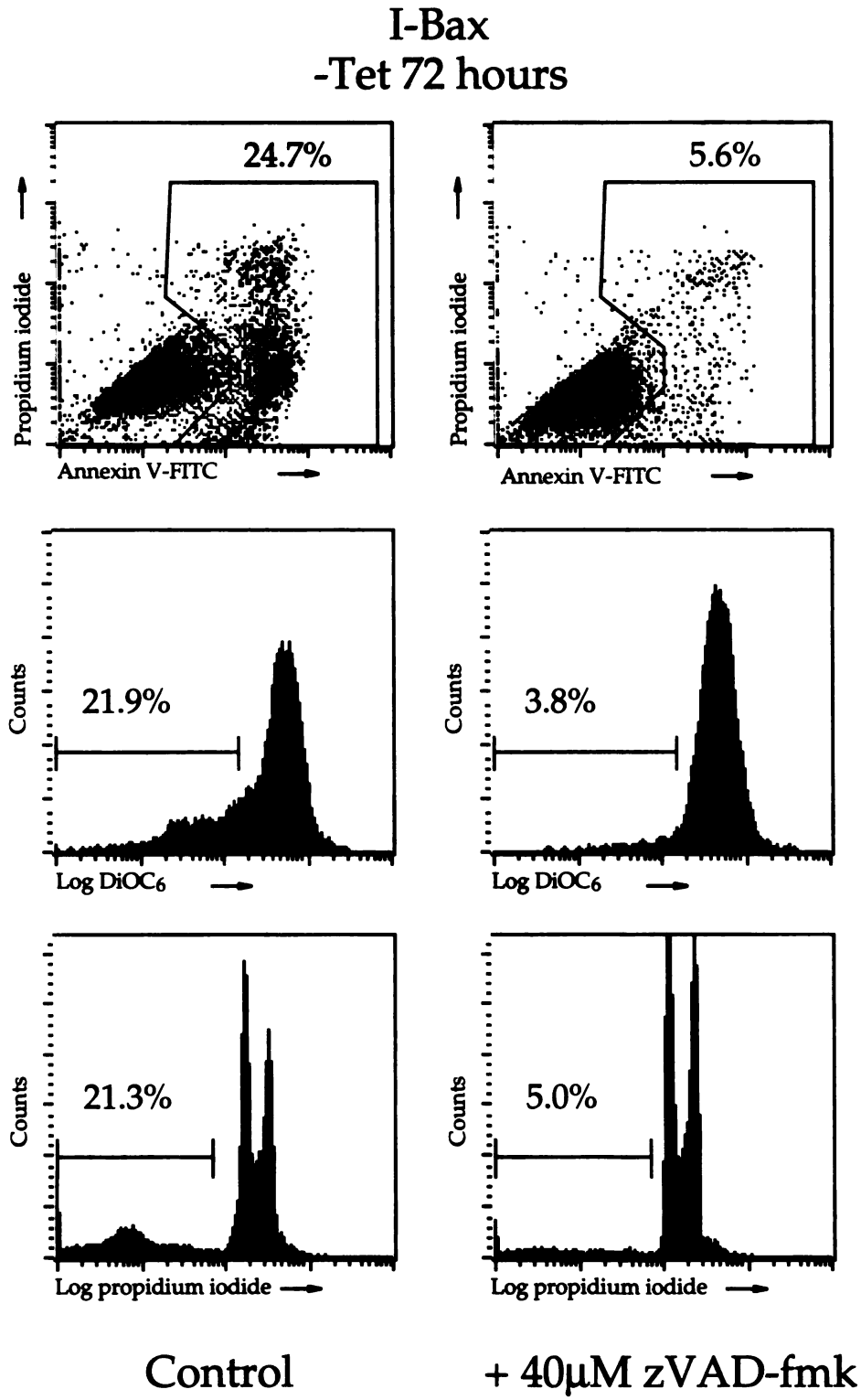
The apoptosis of Tet-deprived I-Bax cells was fully inhibitable by zVAD-fmk, a broad-spectrum caspase inhibitor. As illustrated in Fig. 11A, I found that adding 40 μM zVAD-fmk to the culture medium at the time of Tet withdrawal completely suppressed the apoptotic response, as assayed in flow cytometric assays for surface annexin V binding, for $\Delta\psi_m$ using $DiOC_6$, or for nuclear DNA content. In each of these assays, the percentage of apoptotic cells at 72 hr post-withdrawal was reduced from 22-25% in the absence of zVAD-fmk to 3-6% in its presence (Fig. 11A); the latter values are indistinguishable from background levels seen prior to Tet withdrawal or in parental GM701. Cleavage of PARP was also completely suppressed by zVAD-fmk for over 72 hours (Figure 11B). Using a more sensitive assay based on the fluorescent mitochondrial dye JC-1, I did observe a subtler effect of Bax on mitochondrial function which persisted despite

Figure 11. Caspase inhibition prevents Bax-induced apoptosis of I-Bax cells following withdrawal of Tet. (**A**) Cells were grown for 72 hrs without Tet, in either the absence (left panels) or presence (right panels) of zVAD-fmk. Apoptotic cells were scored by using FACS to assay binding of annexin V-FITC (upper panels), reduction in $\Delta\psi_m$ as measured by $DiOC_6$ fluorescence (middle panels), or the presence of subgenomic DNA contents as measured propidium iodide staining of fixed cells (lower panels). The percentage of apoptotic cells is indicated in each panel.

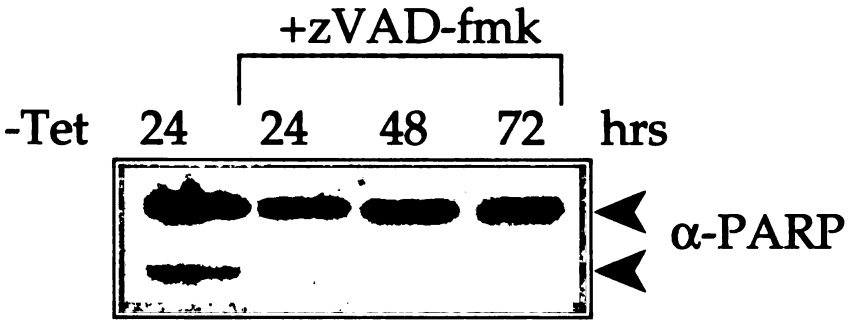
(**B**) PARP cleavage is prevented by zVAD-fmk. Cell lysates were prepared exactly as in Figure 9E. (**C**) zVAD-fmk cannot block all mitochondria changes induced by HABax. I-Bax cells were grown either with Tet or without Tet in the absence or presence of zVAD-fmk, then stained with JC-1 dye and analyzed by FACS to detect JC-1 monomer (x= FL-1) and dimer (y= FL-2) fluorescence. Mitochondrial dysfunction is reflected in a loss of red JC-1 dimer emission.

Figure 11

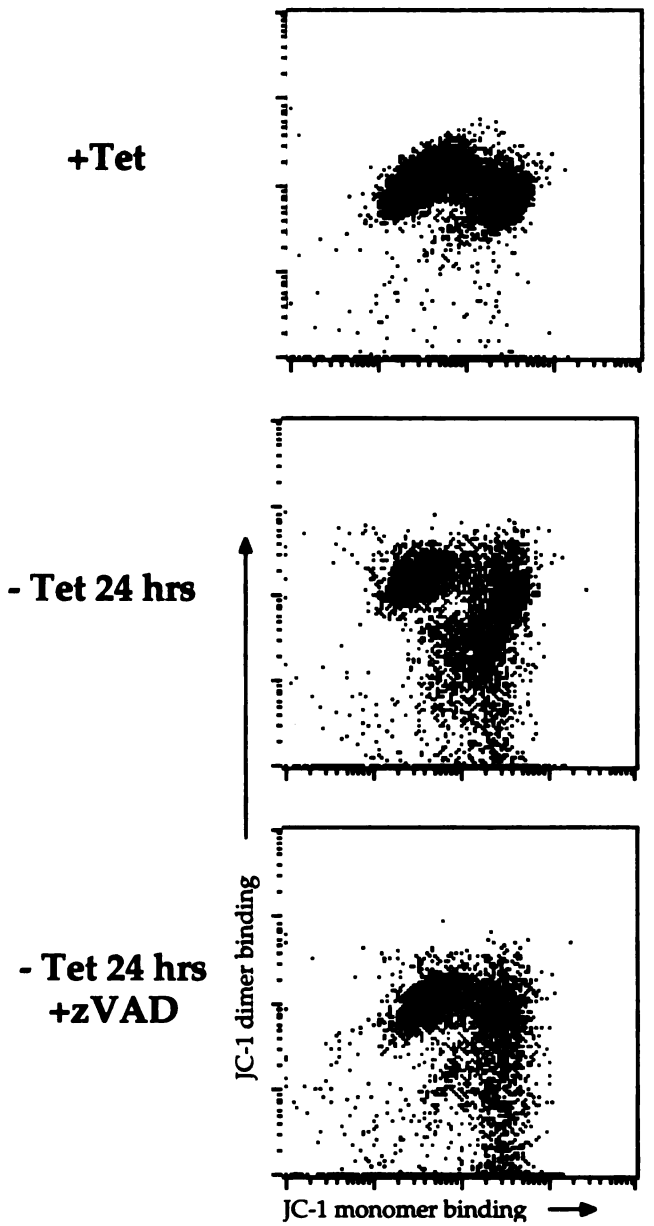
A


I-Bax
-Tet 72 hours
24.7%
5.6%
Propidium iodide
Annexin V-FITC
21.9%
3.8%
Counts
Log DiOC6
21.3%
5.0%
Log propidium iodide
Control
+ 40µM zVAD-fmk

B


+zVAD-fmk
-Tet
24
24
48
72
hrs
α-PARP

C


+Tet
- Tet 24 hrs
- Tet 24 hrs
+zVAD
JC-1 dimer binding
JC-1 monomer binding

caspase inhibition: 24 hr of Tet withdrawal alone caused a significant loss of $\Delta\psi_m$ (reflected in a decrease in red emission from JC-1), which was essentially unaffected by zVAD-fmk (Fig. 11C). Thus, when caspases are inhibited, Bax can cause some perturbation of mitochondrial function in these cells, but fails to open the PT pore or induce apoptosis.

To understand how HABax functions to activate apoptosis, I examined the biochemical characteristics of HABax and other key regulatory molecules in I-Bax cells. I had already observed that repression of the transgene was incomplete, in that there is some HABax expressed when Tet is in the media. I compared the growth kinetics of I-Bax +Tet cells to GM701 or hygro, and found no difference in the rate of cell growth due to the transgenic HABax expression (data not shown). I next tested several apoptotic inducing agents to determine if this exogenous Bax expression caused these cells to be more sensitive to negative stimuli (Figure 12). I observed little difference between GM701, hygro, and I-Bax +Tet cells in their apoptotic responses to either UV irradiation (Fig 12A) or staurosporine treatment (Fig 12B). When I removed the Tet to induce HABax, I consistently observed a synergy between Bax and the additional apoptotic stimuli.

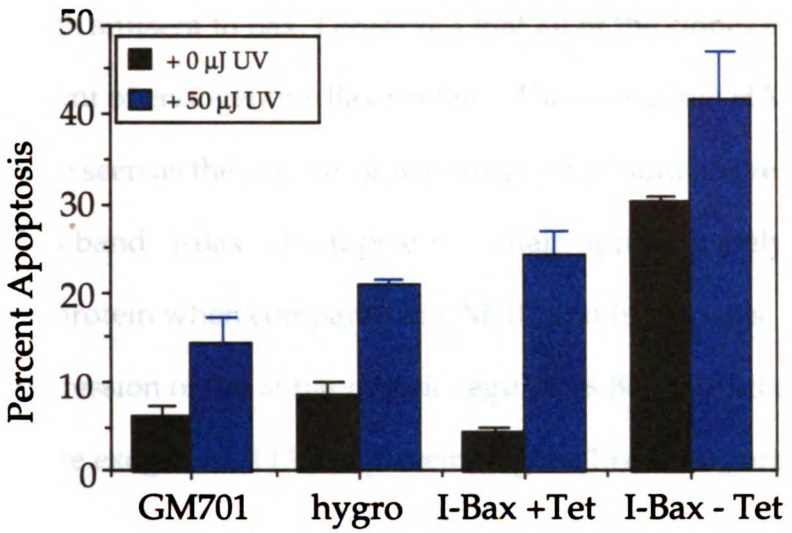
Because I observed no increase in sensitivity to other apoptotic inducing agents, I reasoned that I-Bax cells might compensate for the increase in Bax protein levels by increasing the activity of a negative regulator. I first examined the total amount of Bax in I-Bax cells and compared it to the amount in GM701 and hygro cells to determine if Bax levels in the cell can be regulated at the transcriptional or translational level (Figure 13). I expected the endogenous Bax

Figure 12. Expression of HABax in uninduced I-Bax does not enhance sensitivity to the apoptotic activators UV radiation and staurosporine.

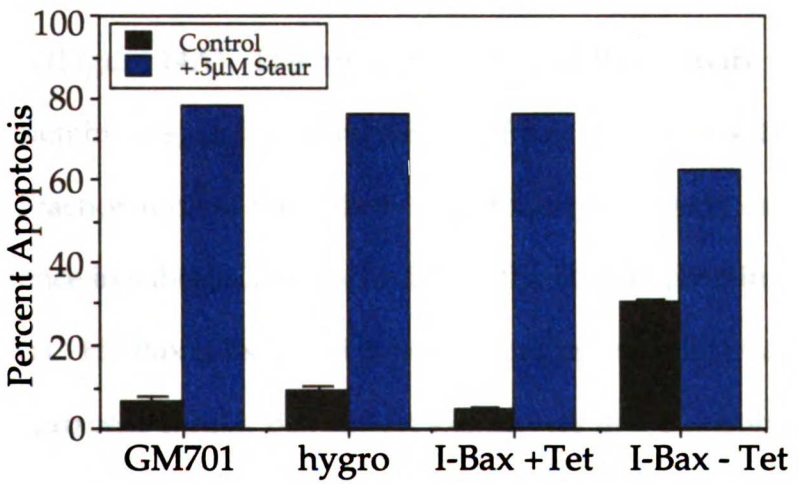
(**A**) Each indicated cell line was irradiated with 50 μJoules of UV from a Stratalinker® (Stratagene). (**B**) Each cell line was treated with .5 μM staurosporine for 16 hours. Apoptosis was assessed by FACS analysis of annexin V-FITC and propidium iodide staining. Results are expressed as the mean of two experiments ± standard deviation.

Figure 12

A


50
40
30
20
10
0
Percent Apoptosis
+ 0 µJ UV
+ 50 µJ UV
GM701
hygro
I-Bax +Tet
I-Bax - Tet

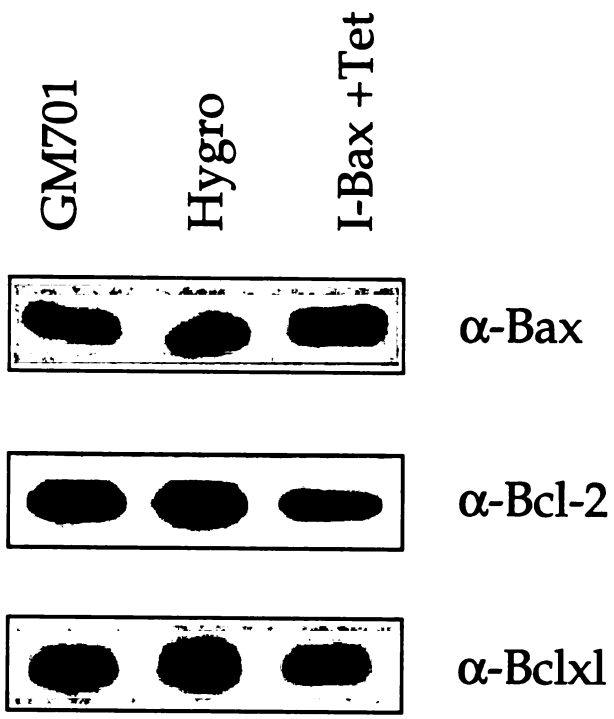
B


100
80
60
40
20
0
Percent Apoptosis
Control
+.5µM Staur
GM701
hygro
I-Bax +Tet
I-Bax - Tet

protein levels to drop if the cell can only tolerate a specific concentration of Bax. Probing total cell lysates from parental GM701, hygro cells, or I-Bax cells by immunoblot with antisera to Bax, I observed that all of the clones expressed a significant amount of endogenous Bax protein. The transgenic HABax in the I-Bax lysate can be seen as the slightly slower-migrating band above the endogenous Bax band. I-Bax cells appear to contain approximately equal levels of endogenous protein when compared to GM701 and hygro cells. I next examined if expression of the antiapoptotic regulators Bcl-2 or Bclxl had altered in response to the exogenous HABax protein (Figure 13). Comparing protein expression levels by immunoblot analysis, I observed no change in expression levels of Bcl-2 or Bclxl among GM701, hygro, or I-Bax + Tet cells. I then examined the cellular distribution of Bax in uninduced I-Bax cells versus GM701 and hygro cells (Figure 14A). The primary location of Bax activity is believed to be at cellular membranes, and so sequestering of Bax in the cytosol would act to protect cells. Fractionation of the cells by hypotonic lysis clearly demonstrated a marked difference in subcellular localization of the HABax protein versus endogenous Bax. HABax is localized primarily in the cytosolic fraction, while GM701 and hygro cells exhibit Bax almost exclusively in the membrane fraction. Uninduced I-Bax cells also have Bax on their membranes, and the majority appears to be endogenous protein, as there is only a small amount visible in the shifted HABax band corresponding to the amount seen in the anti-HA blot. Bclxl can be found in both cellular compartments, and its localization did not differ in response to the cytosolic Bax. I confirmed that the cytosolic fraction did not contain mitochondria by examining the distribution of the inner mitochondrial

Figure 13. Comparison of the apoptotic proteins Bax, Bcl-2 and Bclxl among GM701, hygro, and I-Bax +Tet cells. Total cell lysates were prepared from each cell line. 20μg of each sample was separated by 14% SDS-PAGE, and blotted with the indicated antibody.

Figure 13



membrane protein, cytochrome oxidase (COX). The membrane fraction did contain mitochondria, but because this is a crude fractionation, I cannot verify that Bax is localized exclusively to mitochondria. I also examined the localization of newly synthesized HABax protein after withdrawal of Tet (Figure 14B). A large portion of the new protein appears in the membrane fraction after 48 hours of Tet withdrawal, confirming previous studies that suggest that Bax functions primarily on membranes to induce apoptosis. I concluded that the transgenic Bax is compartmentalized away from the main site of its apoptosis inducing effects, and that is why there is no increased mortality in response to negative stimuli. Increased expression through withdrawal of Tet causes this inhibition to fail and HABax concentration in the membrane fraction increases and thereby induces apoptosis. However, I also observe a significant amount of newly synthesized HABax in the cytosolic fraction (Fig 14B), so I cannot rule out a similar effect of the increased concentration of HABax titrating out a cytosolic regulator, and thereby activating a cytosolic pathway for the induction of apoptosis. As more is learned about the regulation of Bax in membranes, it should be possible to create mutations that disrupt membrane attachment and thereby determine the necessity for this interaction.

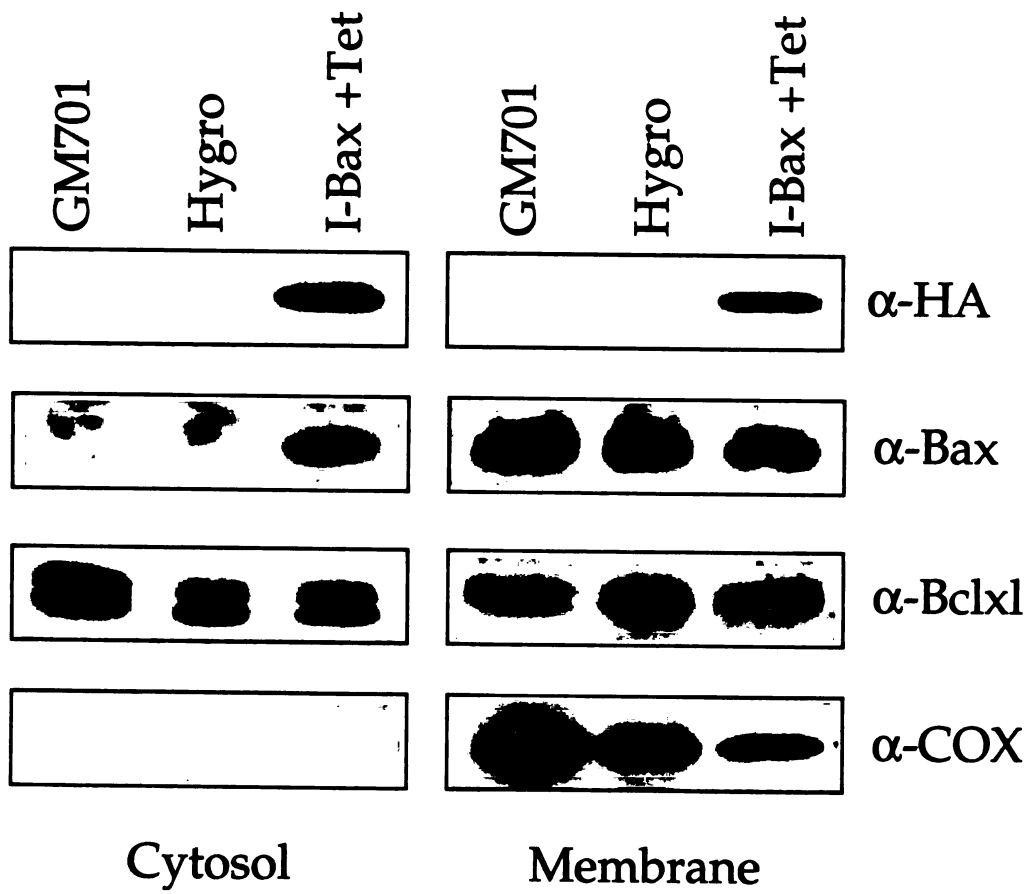
Cytochrome c is an important apoptotic signaling molecule that can activate caspases through the intermediary protein, Apaf-1, and so I examined the subcellular distribution of cyto c in our I-Bax cells with and without Tet. After 24 hours without Tet, cytochrome c can be seen in the cytosol of I-Bax cells (Figure 15). The level of cyto c in the cytosol increases over 48 hours, and then appears to drop off after 72 hours, presumably because many cells have already

Figure 14. HABax localizes to both the cytosol and the membrane.

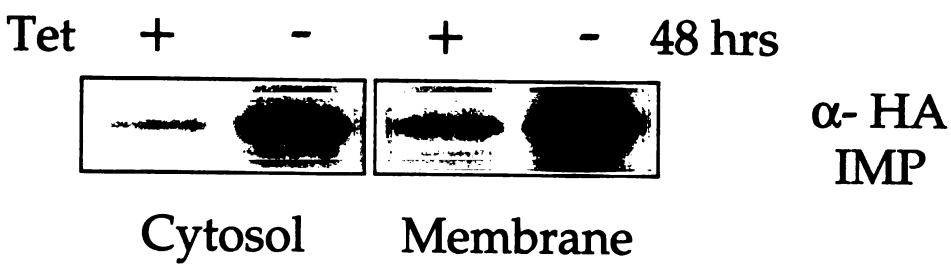
(**A**) Comparison of the subcellular localization of HABax, Bax and Bclxl among GM701, hygro, and I-Bax +Tet cells. Cells were fractionated by isotonic lysis, 20μg of each sample was separated by 14% SDS-PAGE, and blotted with the indicated antibody. (**B**) Newly synthesized HABax localizes to both the membrane and cytosol fraction. 42 hours after the withdrawal of Tet, the media was replaced by methionine-minus media containing 10 μCi/ml ^{35}S-methionine. 6 hours later, isotonic lysates were prepared of the labeled cells and immunoprecipitated with anti-HA, and immune complexes were separated by 14% SDS-PAGE and analyzed by autoradiography.

Figure 14

A


GM701
Hygro
I-Bax +Tet
GM701
Hygro
I-Bax +Tet
α-HA
α-Bax
α-Bclxl
α-COX
Cytosol
Membrane

B

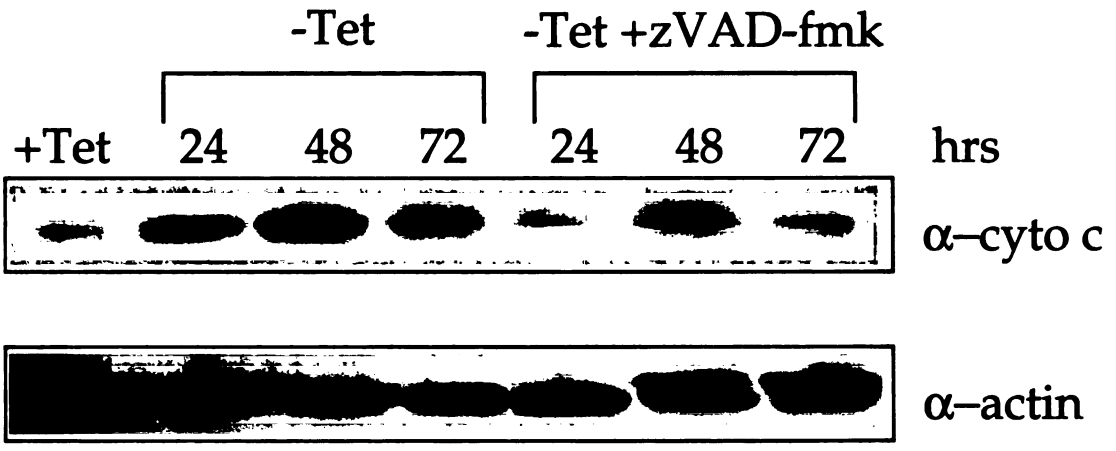

Tet + - + - 48 hrs
α- HA
IMP
Cytosol
Membrane

undergone apoptosis. I also examined the effect of caspase inhibition on the distribution of cyto c. After 24 hours without Tet and with zVAD-fmk, cyto c was detected in the cytosolic fraction. Its presence increased after 48 hours, but had considerably diminished after 72 hours. I do not understand why cyto c disappears from the cytosol by 72 hours, as no cells are dying, but it suggests there is a regulatory mechanism that removes proteins that are in an improper cellular location. These results confirm the JC-1 results shown above, that although zVAD-fmk can block the outward manifestations of apoptosis, mitochondria are affected by the induction of HABax. This also demonstrates that cyto c can translocate from the mitochondria without the opening of a megachannel, as the $DiOC_6$ data in Figure 11A show that the mitochondria of I-Bax -Tet + zVAD-fmk cells have not lost their membrane potential as would be expected if a megachannel had opened.

To better understand the effect of HABax on the mitochondria, I also examined individual cells using indirect immunofluorescence. HABax was not detectable in uninduced cells, nor in many cells after withdrawal of Tet, presumably because it is diffuse in the cytosol. However, 24 hours after withdrawal of Tet, HABax can be seen localizing to discrete structures (Figure 16A), which I verified were mitochondria by staining for cyto c and Mitotracker Green, a mitochondria-specific fluorescent dye. I also observed HABax binding to mitochondria which were cyto c negative, and this release was not prevented when zVAD-fmk was present, confirming the biochemical results. Mitochondria are normally spread throughout the cytoplasm, but many HABax positive cells exhibited a reorganization of the mitochondria to a perinuclear arrangement.

Figure 15. Bax induced cytochrome c release from mitochondria is caspase independent. Cytosolic fractions were prepared from cells which had been induced by Tet withdrawal with and without 40 μM zVAD-fmk as indicated. 20μg of lysate was separated by 14% SDS-PAGE and immunoblotted with the indicated antibody.

Figure 15



Cytosol

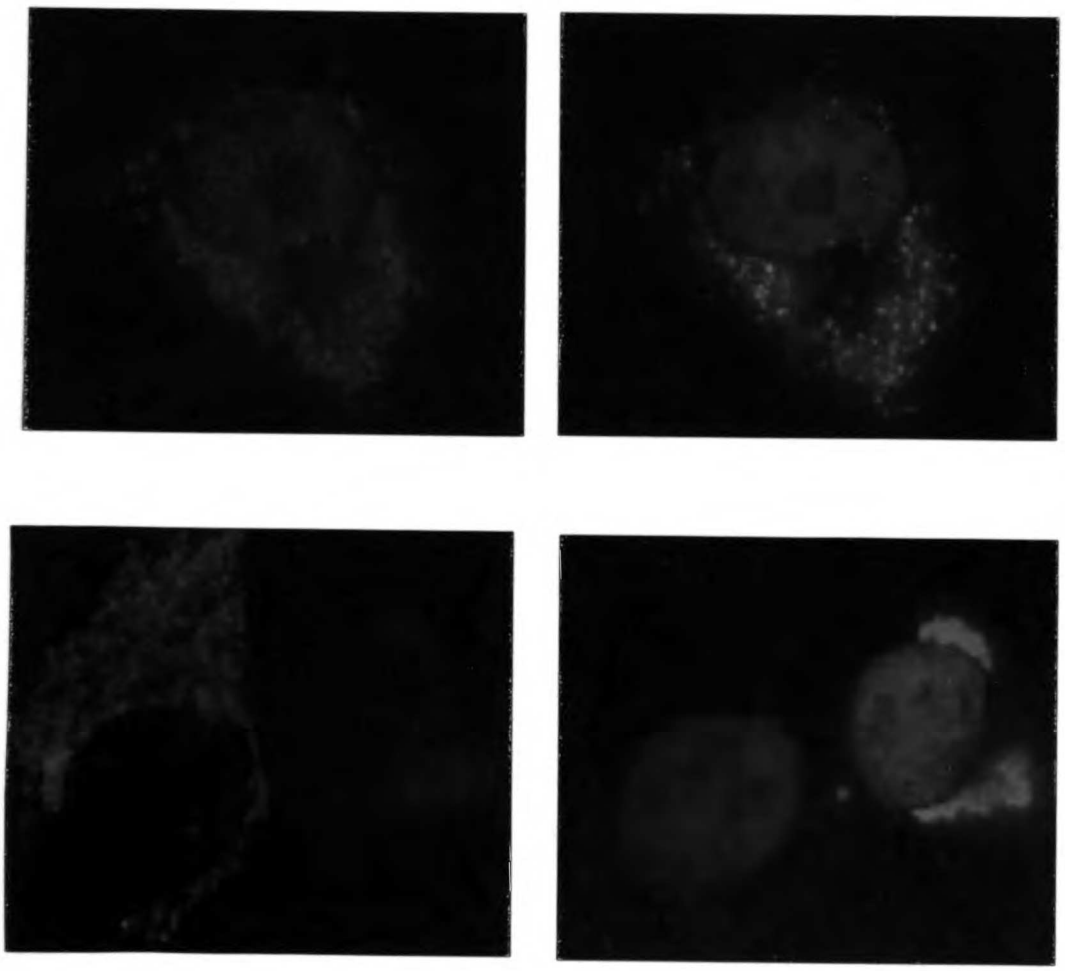
This mitochondrial clustering was also caspase independent, as this pattern did not change when zVAD-fmk was added. I believe that the sequence of HABax induced mitochondria derangement is initiated with HABax binding to the mitochondria, release of cyto c, and then clustering of mitochondria around the nucleus, as this was the predominate organization observed at 72 hours post-Tet withdrawal (Figure 16B). I do not observe cyto c in HABax positive cells by immunofluorescence at this time point, possibly because it is diffuse in the cytoplasm and below detection, or because it has been sequestered by an unknown mechanism, as was suggested from the biochemical data.

Figure 16. Induction of HABax by Tet withdrawal causes Bax to associate with mitochondria and induce the release of cytochrome c via a caspase independent mechanism. (**A**) I-Bax cells plus 40μM zVAD-fmk were examined 24 hr after Tet-withdrawal, then fixed and double labeled, first with cytochrome c visualized by anti mouse-FITC (green), then by rhodamine-conjugated anti-HA (red). The nuclei were visualized by DAPI (blue). Each pair of micrographs depicts a single field showing cyto c (at left) or HA and nuclear staining (at right). (**B**) I-Bax cells 72 hours after Tet withdrawal. Image is a composite of all three fluorescent indicators.

Figure 16

A

I-Bax
-Tet 24 hours

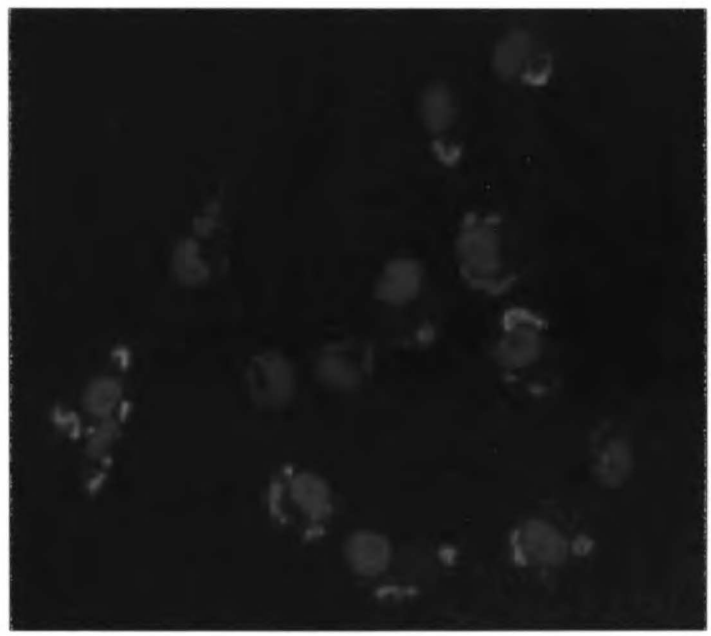


cytochrome c

HA/DAPI

B

- Tet 72 hrs



CONCLUSIONS

I have established a tetracycline repressible expression system in which to study the effects of Bax-induced apoptosis. The cell line I chose to use is the human fibroblast line GM701, which had previously been shown to undergo rapid apoptosis when transiently transfected with a gene encoding Bax. I confirm these effects by showing that induction of Bax in the I-Bax cell line causes apoptosis that can be observed within 12 hours. I also confirm the finding from the transient transfection studies described in Chapter Two that caspase inhibition by zVAD-fmk prevents Bax induced apoptosis in GM701 fibroblasts. This is in contrast to two previous reports which found that Bax induced apoptosis did not require caspase activation. In those studies, which employed the T lymphocyte line Jurkat, overexpressing Bax alone was sufficient to cause rapid cell death that occurred despite treatment with zVAD-fmk at dosages that effectively blocked both Bax-induced caspase activation and Fas-mediated apoptosis (69,116). This suggests that, in Jurkat cells, Bax simultaneously activates two parallel pathways—one caspase-dependent, the other caspase-independent—that can each lead independently to death. A likely interpretation for this discrepancy is that there are important tissue-specific differences in the regulation of the two pathways. Consistent with this view, earlier studies of *caspase 9*-deficient mice found that thymocytes from such animals were sensitive to radiation-induced apoptosis but that embryonic fibroblasts were not (103,108). This suggests that T cells are more sensitive to an alternative death pathway that does not require caspases than are fibroblasts.

I propose this cell line to be a model system in which to study Bax

induced apoptosis. To this end, I have determined that Bax can induce the release of cyto c from mitochondria and a relocalization of mitochondria without the activation of caspases or the opening of the PT pore. It is not yet understood if Bax directly causes these changes in mitochondria, possibly through a regulatory pore that differs from the PT pore, or if it interacts with another molecule to activate these changes, and use of this cell line should aid in the delineation of these events. Another key question will be to determine what the differences are between GM701 fibroblasts and Jurkat cells which enable one to survive Bax induced apoptosis when caspases are inhibited, whereas the other cannot. I have described a difference in subcellular distribution of endogenous Bax and the transgenic HABax in this cell line. The difference between HABax and endogenous Bax of an epitope tag at the N-terminus suggests an unknown regulatory domain may be located in this region that is interrupted by the additional amino acids of the epitope tag. Further study is needed to determine if this is the reason for the mislocalization, and if so, to determine how this N-terminal domain functions to ensure the proper localization of Bax.

CHAPTER FOUR

BAX AND A COMPONENT OF THE MITOCHONDRIA SYNERGIZE TO INDUCE CASPASE INDEPENDENT APOPTOSIS

Apoptosis has been observed in many different species, and it can be initiated through a wide variety of signals, including growth factor withdrawal, cytotoxic damage, hormone treatment, and developmental program. Strikingly, these diverse pathways result in activation of a similar suicide program within the cell. Regulatory molecules, such as caspases and Bcl-2 family proteins, have been shown to function across diverse species barriers, suggesting an evolutionarily conserved pathway of apoptosis (11,16). Along with the biochemical data suggesting a role of mitochondria in cell death, the conservation between eukaryotes of many components of the mitochondria is another compelling reason to believe that these organelles may be key components in the initiation and commitment to apoptosis.

For many years, ROS production was believed to be the initiating signal from the mitochondria that was responsible for many types of apoptosis (27). ROS were observed to appear soon after addition of a glucocorticoid analog to T cells, as well as in cells treated with tumor necrosis factor (TNF) (21). Overexpression of Bcl-2 was shown to repress this production of ROS, and addition or depletion of anti-oxidants was also shown to inhibit or activate apoptosis respectively (14,40). However, certain types of apoptosis did not appear to cause ROS production, and so a controversy arose concerning the role

of ROS. Arguments were made that although they were not detected, locally high concentrations of ROS could be produced that caused the induction of apoptosis. To address this issue, cells termed ρ^0 were made which lacked mitochondrial DNA and therefore could not produce ROS because they were not undergoing respiration. These ρ^0 cells underwent apoptosis in response to several stimuli, and it was further demonstrated that Bcl-2 overexpression protected these cells (13). Apoptosis was also shown to occur in an anoxic environment (very low O_2 conditions) and this cell death was also inhibitable by Bcl-2 (44). These experiments suggested that ROS were a product of the cell death process, and not a critical signal produced by the mitochondria to initiate apoptosis.

It was about this time that caspases were being characterized, and so the study of mitochondrial activation of apoptosis was all but discarded. However, a few researchers continued their investigations of mitochondria, and described several characteristics of mitochondria in dying cells. It was demonstrated conclusively that mitochondria lost their membrane potential ($\Delta\psi_m$) before nuclear events of apoptosis had begun, and that this loss preceded production of ROS. To further prove a mitochondrial function in apoptosis, Kroemer and his colleagues created ρ^0 cells in a cell line which was sensitive to tumor necrosis factor (TNF)-induced apoptosis, and showed that these cells maintain a $\Delta\psi_m$ that is lost during TNF induced death (59). They also described a soluble factor, which is sequestered in normal and ρ^0 mitochondria, that was released from mitochondria during apoptosis and could induce apoptotic effects when added to normal nuclei. Soon after these studies, Wang described how cyto c

translocates from the mitochondria to the cytosol early in apoptosis, and so there was renewed attention to the mitochondrial regulation of apoptosis.

Understanding the role of mitochondria in the induction of apoptosis requires an insight into the normal biology of respiring mitochondria (20). The $\Delta\psi_m$ is created by the three complexes of the electron transport chain, which pump protons out of the matrix by the energy created from passing electrons from a high-energy state to a lower state. The primary function of $\Delta\psi_m$ is to power the conversation of ADP to ATP by the F_1F_0ATPase, a large complex of proteins that form a channel in the inner mitochondrial membrane. Maintenance of the $\Delta\psi_m$ also requires the permeability of the two mitochondrial membranes to be tightly regulated. The inner mitochondrial membrane is completely impermeable to ions and proteins. Any component that must enter or exit the matrix uses a specific channel so not to disrupt the $\Delta\psi_m$. The outer mitochondrial membrane contains constitutively open pores that allow the free flow of ions, but allow nothing larger than 5000 daltons to enter the intermembranous space, and so most proteins must be specifically imported, and by analogy specifically exported if they are to leave the mitochondria.

Loss of $\Delta\psi_m$ destroys the major source of ATP for a cell, and therefore contributes to the death of the cell. Besides cytochrome c, several other factors have been identified that are released by mitochondria during apoptosis. Apoptosis-initiating factor (AIF) (132) has been shown to translocate to the nucleus during apoptosis to activate nuclear breakdown, and caspases-2 and -9 are also released from mitochondria during apoptosis (131). It is not fully understood how these proteins translocate or the $\Delta\psi_m$ is lost, but one possibility

is through the opening of a megachannel. Using purified mitochondria from apoptotic cells, this channel, which is called the permeability transition pore (PT), has been partially characterized, and appears to be composed of multiple proteins, most of which are unknown. Several reports have demonstrated that the adenine nucleotide transport protein (ANT) and cyclophilin D are part of this complex, and inhibition of either of these pharmacologically can inhibit opening of the PT in an *in vitro* assay system (70,119). Cyto c and AIF translocation have been shown to occur with the opening of the PT, and inhibition of caspases or over-expression of Bcl-2 have also been shown to block the opening of the PT *in vivo* (64), suggesting a relevance of this pore to the commitment to apoptosis. However, I and others have demonstrated that cyto c is released when the PT is not open (98,127), and so there must be a second mechanism by which cyto c and perhaps other apoptotic inducing agents are released from the mitochondria.

In Chapter three, I described a cell line, I-Bax, that inducibly expresses the proapoptotic protein Bax after Tet withdrawal, and which undergoes apoptosis upon induction of Bax. I have used this cell line to further characterize the effects of Bax on the mitochondria. I find that two mitochondria toxins, oligomycin and antimycin, augment Bax induced apoptosis. I also find that synergy between Bax and oligomycin causes caspase independent cell death in GM701 fibroblasts, whereas apoptosis induced by Bax alone, or by Bax and antimycin, is inhibited by caspase inhibition. I have mapped this oligomycin sensitive domain to the BH3 domain, and further demonstrate that point mutations within this region abrogate this effect. This suggests Bax may interact with a specific component of the mitochondria to induce this effect.

MATERIALS AND METHODS

Cell Lines

The human GM701 fibroblast line and all clones derived from it were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamate (2 mM) (UCSF Cell Culture Facility) and 10% (v/v) fetal calf serum (Gemini Biosystems). All cells were maintained at 37°C in 5% CO_2 at 90% relative humidity. All Tet-dependent clones were supplemented with 5 μg/ml Tet every 1-2 days. To remove Tet, cells were washed in phosphate-buffered saline (PBS), trypsinized, and washed again in PBS before replating in fresh media.

Reagents

Rhodamine-conjugated anti-HA (12CA5) and FITC-conjugated goat anti-mouse sera were purchased from Boehringer Mannheim. Anti-human PARP (clone C2-10) and anti-cytochrome c (7H8.2C12) were purchased from Pharmingen. Antibodies were used at dilutions recommended by the suppliers. Propidium iodide, oligomycin, antimycin, and saponin were from Sigma; tetracycline and annexin V-FITC were from Calbiochem; zVAD-fmk was from Alexis Laboratories; $DiOC_6$ and Mitotracker Green were from Molecular Probes.

Western Blot Analysis

Cells were lysed in Triton-X lysis buffer as described in Chapter Three, 50μg of each sample was separated by 8% SDS-PAGE, transferred to hybond paper, then incubated for 1 hr with anti-PARP at a 1:2000 dilution in Blotto. Filters were then washed three times in PBS with 0.1% Tween-20 for 10 min each, incubated with secondary anti-mouse (1:10000) in Blotto for 1 hr, washed again three times, and

developed using ECL reagent (Pierce).

Assessment of Apoptosis by Fluorescence Activated Cell Sorting (FACS)

Both floating and adherent cells were analyzed in all assays. For annexin V binding, cells were harvested using trypsin and then incubated with annexin V-FITC according to the manufacturer's instructions. For analysis of mitochondrial function, 50 nM $DiOC_6$ was added to the media, and cells were harvested 15 min later. All samples were analyzed using a Becton-Dickinson FACSort and Cellquest software, examining a minimum of 10,000 cells in each sample.

Transient Transfection Studies

To create chimeras SW1-SW4, a HindIII restriction site was introduced into basepairs 135-138 of a pSFFV-HABax/β–galactosidase expression plasmid, resulting in missense mutation of threonine to serine at Bax residue 46. Fragments of *bcl2* were cloned into *bax* by PCR using this HindIII site and the Pst I site at basepair 226. Double alanine mutations were also introduced by PCR. Transient transfections were performed as described in Chapter 2. Where indicated, zVAD-fmk and oligomycin were added 6 hr after transfection.

Indirect Immunofluorescence

Cells grown on glass cover slips were transfected as described above, and fixed in 2% paraformaldehyde in PBS. Cells were washed in PBS with 0.03% saponin, then permeablized in PBS with 0.1% saponin and 1% BSA. Cells were double-labeled first with anti-cytochrome c at 1:75 dilution and FITC-conjugated goat anti-mouse IgG at 1:50 dilution, and then with rhodamine-conjugated anti-HA at 1:20 dilution. DAPI was added in the mounting medium (Vectashield, Vector Laboratories). Cells were examined using a Nikon Eclipse TE300 microscope and

IP Lab Spectrum software (Scanalytics, Inc.).

RESULTS

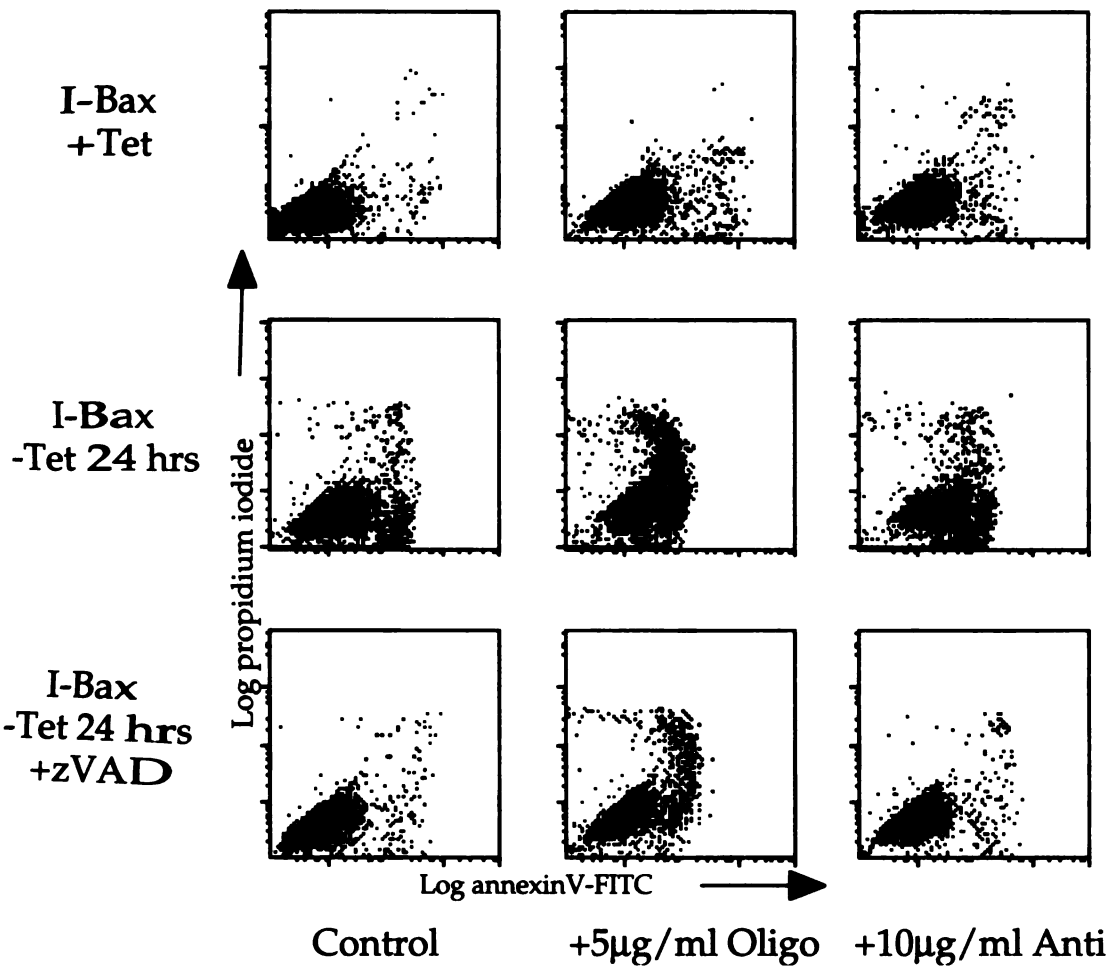
Previous characterization of I-Bax cells had determined that Bax could induce the release of cyto c from mitochondria and a relocalization of mitochondria without the activation of caspases. However, this is not sufficient to induce apoptosis, as cells could be protected by zVAD-fmk for up to 72 hours post Tet withdrawal. Immunofluorescence studies of induced I-Bax cells had demonstrated marked changes to the mitochondria in response to HABax which were caspase independent, and so I asked whether other substances that targeted the mitochondria could function synergistically with Bax. In particular, I tested the effects of two potent mitochondrial toxins -- oligomycin and antimycin -- which inhibit the F_1F_0ATPase and complex III of the electron transport chain, respectively. Typical results are shown in Figure 17. At the concentrations tested, each agent by itself produced little or no increase in death of I-Bax cells growing in Tet, or of Hygro cells, as measured by surface annexin V binding and by staining with propidium iodide (Figs. 17A and 17C). However, each toxin greatly increased the death response that occurred after Tet withdrawal in I-Bax cells, indicating that each could augment the effect of Bax. The death response induced by the combination of Bax and antimycin could be almost completely suppressed by treatment with zVAD-fmk. Remarkably, however, zVAD-fmk yielded only a partial decrease in the response to Bax and oligomycin, so that a substantial rate of cell death was induced with this combination despite caspase

Figure 17. Mitochondria toxins augment HABax-induced apoptosis and can activate a caspase-independent apoptotic pathway. Cells were grown for 24 hr in the presence or absence of Tet and of the mitochondrial toxins oligomycin (Oligo, at 5 μg/ml) or antimycin (Anti, at 10 μg/ml), then assayed for apoptosis by FACS. Where indicated,40μM zVAD-fmk (zVAD) was added at the time of Tet withdrawal. (**A**) Oligomycin and antimycin each augment HABax-induced apoptosis, as detected by surface annexin V-FITC binding. I-Bax cells were grown under the conditions shown, then analyzed for annexin V-FITC binding (x=FL-1) and uptake of propidium iodide (y=FL-2). "Control" denotes no added toxin. (**B**) Mitochondrial toxins augment mitochondrial dysfunction as assayed by $DiOC_6$ binding (x=FL-1). (**C**) Caspase-independent induction of annexin V-FITC binding by the combination of oligomycin treatment and Tet-withdrawal. The percentage of surface annexin V-binding cells was assayed as in panel A, either for Hygro cells (Hygro) or for I-Bax cells under the conditions indicated, in triplicate experiments. (**D**) Caspase-independent induction of mitochondrial dysfunction by the combination of oligomycin treatment and Tet-withdrawal. Cells grown under the indicated conditions were assayed for reduced $\Delta\psi_m$ as described for Fig. 3A, in triplicate experiments.

(E) Fluorescence micrograph of two I-Bax cells, grown for 24 hr after Tet-withdrawal in the presence of zVAD-fmk and oligomycin, stained with the nuclear dye DAPI (blue) and with an HA-specific primary antibody (not shown). The upper, HA-positive cell, shows nuclear and cytoplasmic condensation and is detaching from the plate; the lower, HA-negative cell is shown for comparison. **(F)** PARP is not cleaved during Bax/oligomycin induced caspase independent cell death. Total cell lysates were prepared from the cells which were treated as indicated for 24 hours. 50μg of each sample was separated by 8% SDS-PAGE and blotted with anti-PARP. Arrows denote the 115-kD full-length PARP (upper) and 85-kD cleavage product (lower).

Figure 17

A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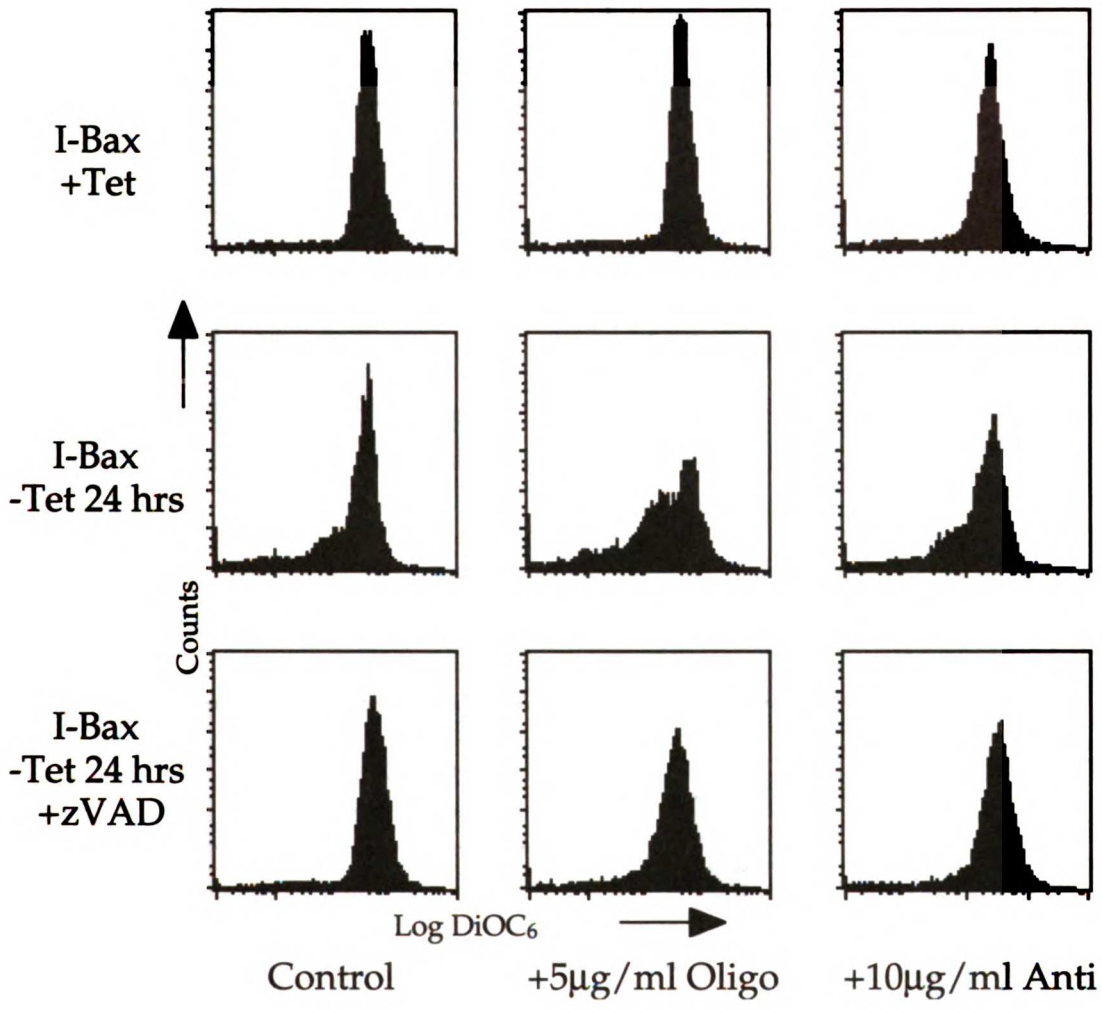
+Tet
I-Bax
-Tet 24 hrs
I-Bax
-Tet 24 hrs
+zVAD
Log propidium iodide
Log annexinV-FITC
Control
+5µg/ml Oligo
+10µg/ml Anti

B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+Tet
I-Bax
-Tet 24 hrs
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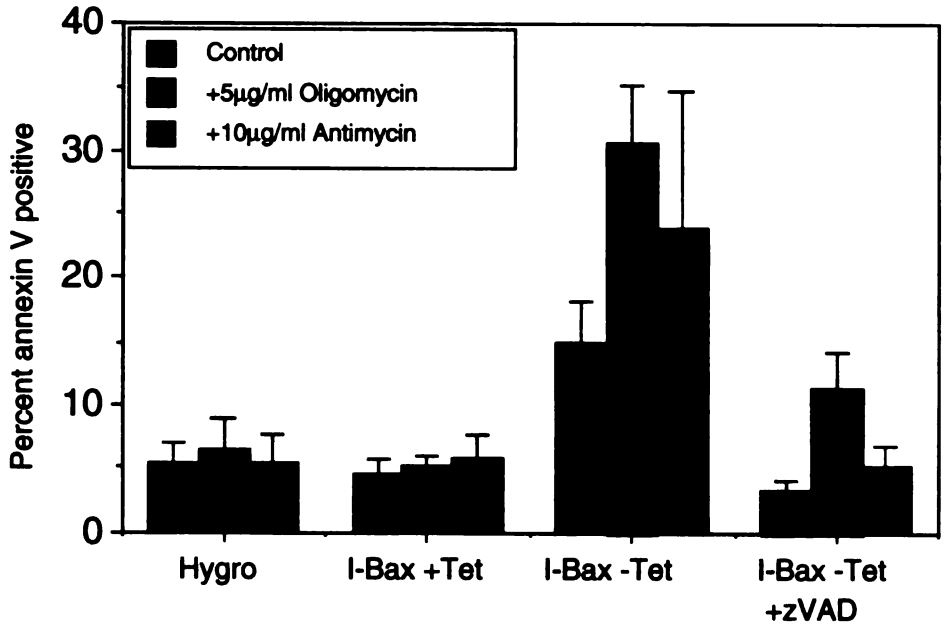
-Tet 24 hrs
+zVAD
Counts
Log DiOC6
Control
+5µg/ml Oligo
+10µg/ml Anti

C


Control
+5µg/ml Oligomycin
+10µg/ml Antimycin
Percent annexin V positive
40
30
20
10
0
Hygro
I-Bax +Tet
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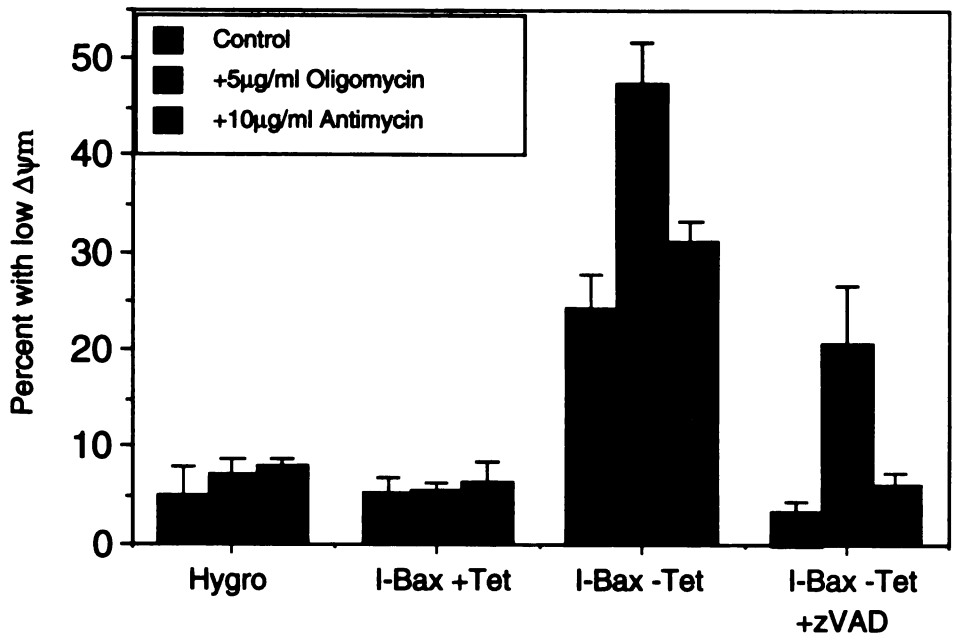
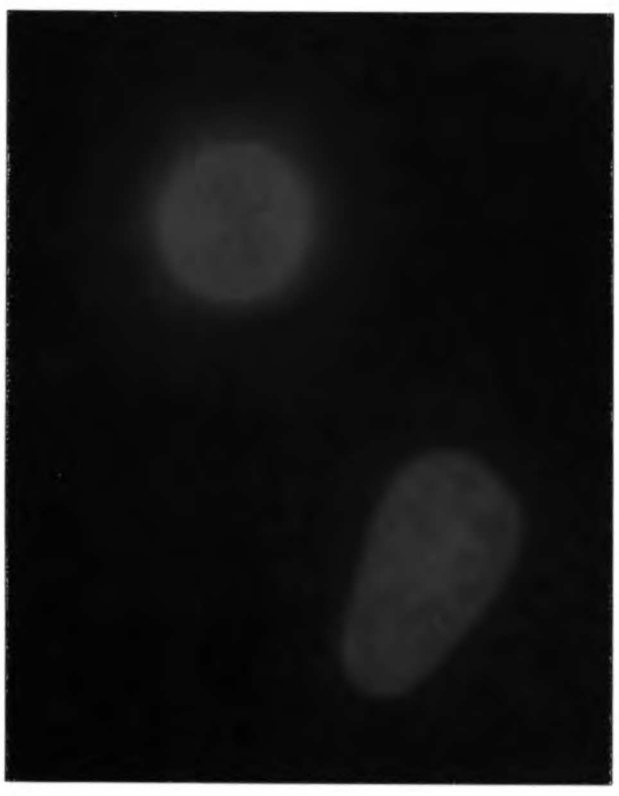
I-Bax -Tet
+zVAD

D


Control
+5µg/ml Oligomycin
+10µg/ml Antimycin
Percent with low Δψm
50
40
30
20
10
0
Hygro
I-Bax +Tet
I-Bax -Tet
I-Bax -Tet
+zVAD

E



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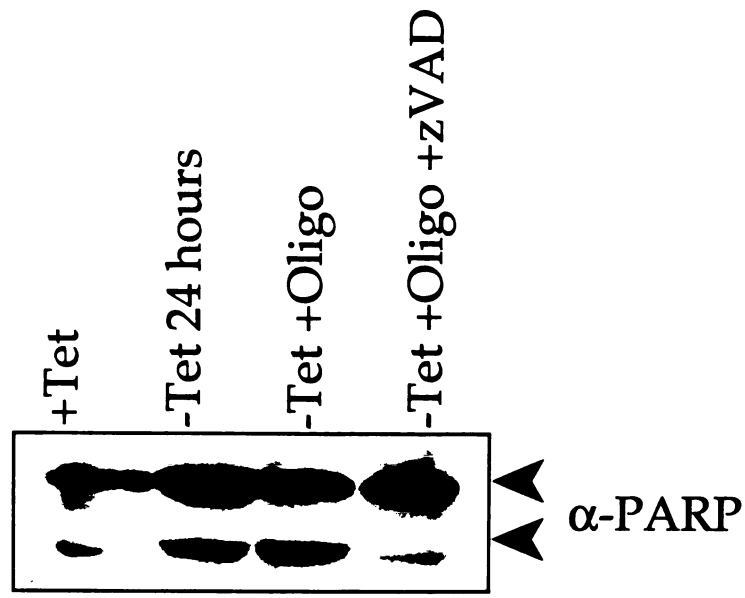


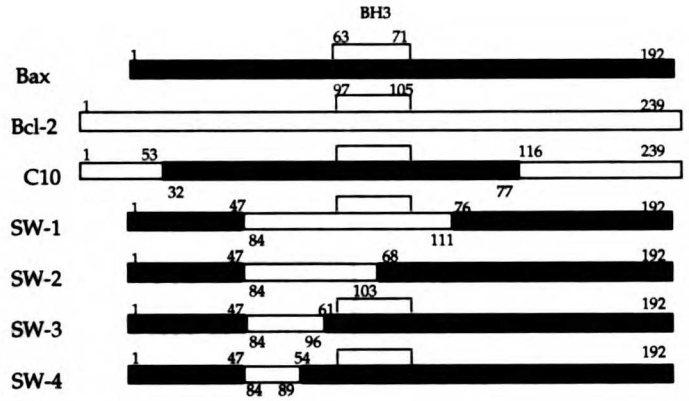
blockade (Fig. 17A and 17C). A similar effect was seen in mitochondria, where the combination of Tet withdrawal and oligomycin was uniquely able to provoke a loss of $\Delta\psi_m$ as measured using $DiOC_6$, despite treatment with zVAD-fmk (Fig. 17B and 17D). Microscopic examination of the dying cells revealed that they failed to exclude trypan blue (data not shown) and that they showed the loss of anchorage, chromatin condensation, nuclear pyknosis, and cytoplasmic shrinkage typical of apoptosis (Fig. 17E). We verified the lack of protease activation by examining cleavage of PARP at 24 hours after treatment with oligomycin and zVAD-fmk (Fig. 17F). These findings therefore define a caspase-independent pathway of cell death that yields many of the characteristic changes of apoptosis and is triggered by the combined effects of oligomycin and Bax.

In Chapter two, I demonstrated that Bax can induce apoptosis in both a BH3 dependent and independent manner, and so therefore I asked whether the BH3 domain of Bax was required for caspase-independent killing. To address this question, I constructed a series of plasmids encoding HA-tagged chimeric proteins (Fig. 18A) formed from selected regions of Bax (filled bars) and Bcl-2 (open bars), one of which was previously described in Chapter Two as Bax/Bcl-2, but will be referred to herein as SW-1. The ability of these proteins to induce apoptosis independently of oligomycin was tested by using the same transient transfection system in GM701 fibroblasts that was described in Chapter two. As shown previously, Bax itself induces apoptosis in approximately 55% of cells, well above the background level obtained with Bcl-2. As previously described (55), a chimera termed C10, in which the BH3 domain of Bcl-2 is replaced by that of Bax, also induces apoptosis efficiently. For this study, I created three

Figure 18. The BH3 domain of Bax is required for synergy with oligomycin. (**A**) Schematic view of Bax (filled bars), Bcl-2 (open bars), and four chimeric proteins tested in this study. Key residues are numbered above and below each construct. Complete BH3 domains, where present, are indicated by brackets; SW-2 contains portions of the BH3 domains of both Bax and Bcl-2. All of these constructs carried an N-terminal HA-epitope tag (not shown). (**B**) Apoptogenic activities of the tested constructs. Plasmids encoding the indicated proteins were transiently transfected along with a β–gal expression plasmid into GM701 cells, which were then scored morphologically for apoptosis at 18 hr post-transfection. Data shown are mean ± standard deviation for three assays. (**C**) Caspase-independent apoptosis. GM701 cells were transiently transfected with the indicated constructs, then treated 6 hr later with zVAD-fmk with or without oligomycin, and then assayed for apoptosis as in panel B, at 28 hr post-transfection. Data shown are mean ± standard deviation for four assays.

Figure 18

A


BH3
63
71
Bax
1
192
97
105
Bcl-2
1
239
C10
1
53
116
239
32
77
SW-1
1
47
76
192
84
111
SW-2
1
47
68
192
84
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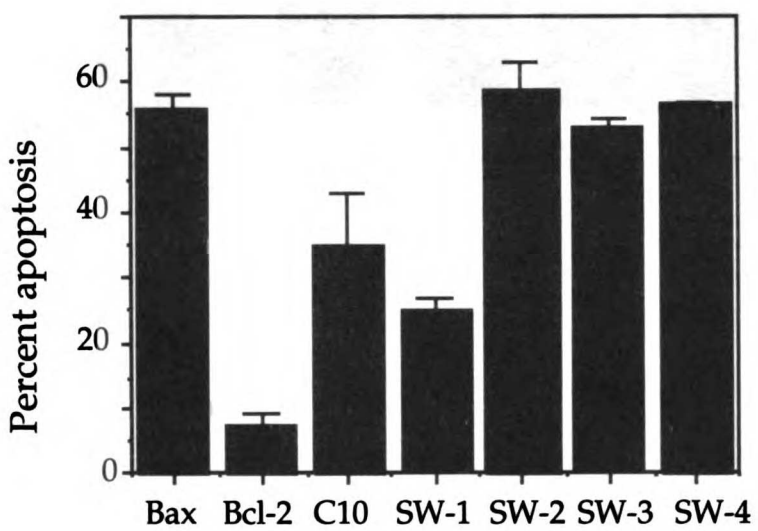
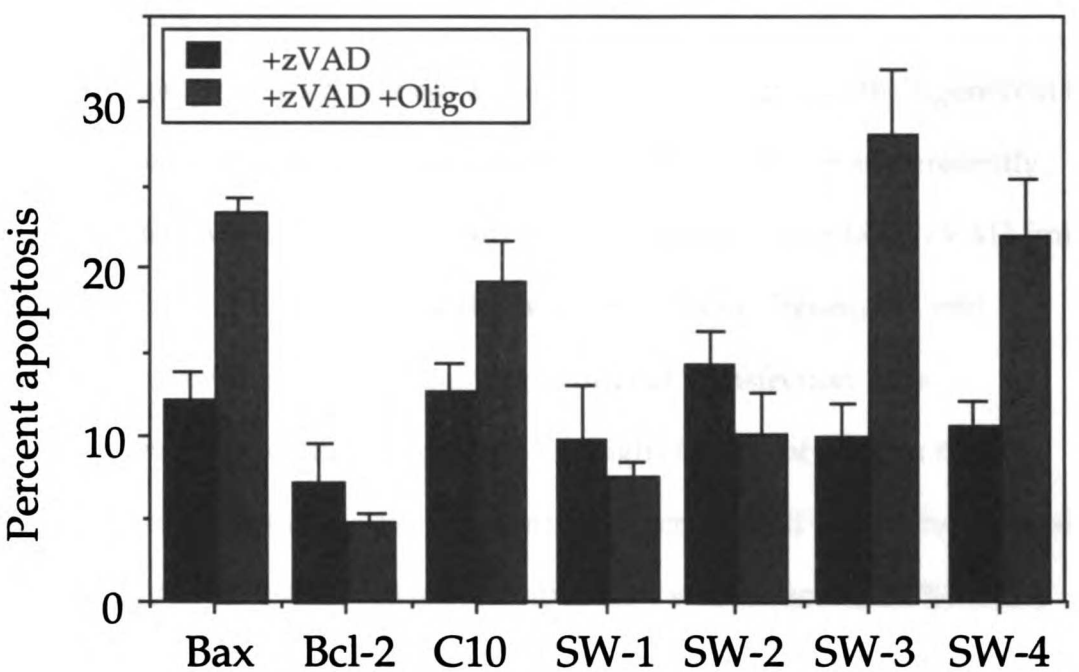
1
47
61
192
84
96
SW-4
1
47
54
192
84
89

B


Percent apoptosis
60
40
20
0
Bax
Bcl-2
C10
SW-1
SW-2
SW-3
SW-4

C


+zVAD
+zVAD +Oligo
Percent apoptosis
0
10
20
30
Bax
Bcl-2
C10
SW-1
SW-2
SW-3
SW-4

additional chimeras like SW-1 in which progressively shorter sequences in and around the BH3 region from Bcl-2 replaced the corresponding sequences in Bax; two of these (SW-3 and SW-4) contained the complete Bax BH3 domain, but the other two (SW-1 and SW-2) did not. All of the latter chimeras had proapoptotic activities comparable to that of Bax itself, except for SW-1, which was roughly half as active (Fig. 18B).

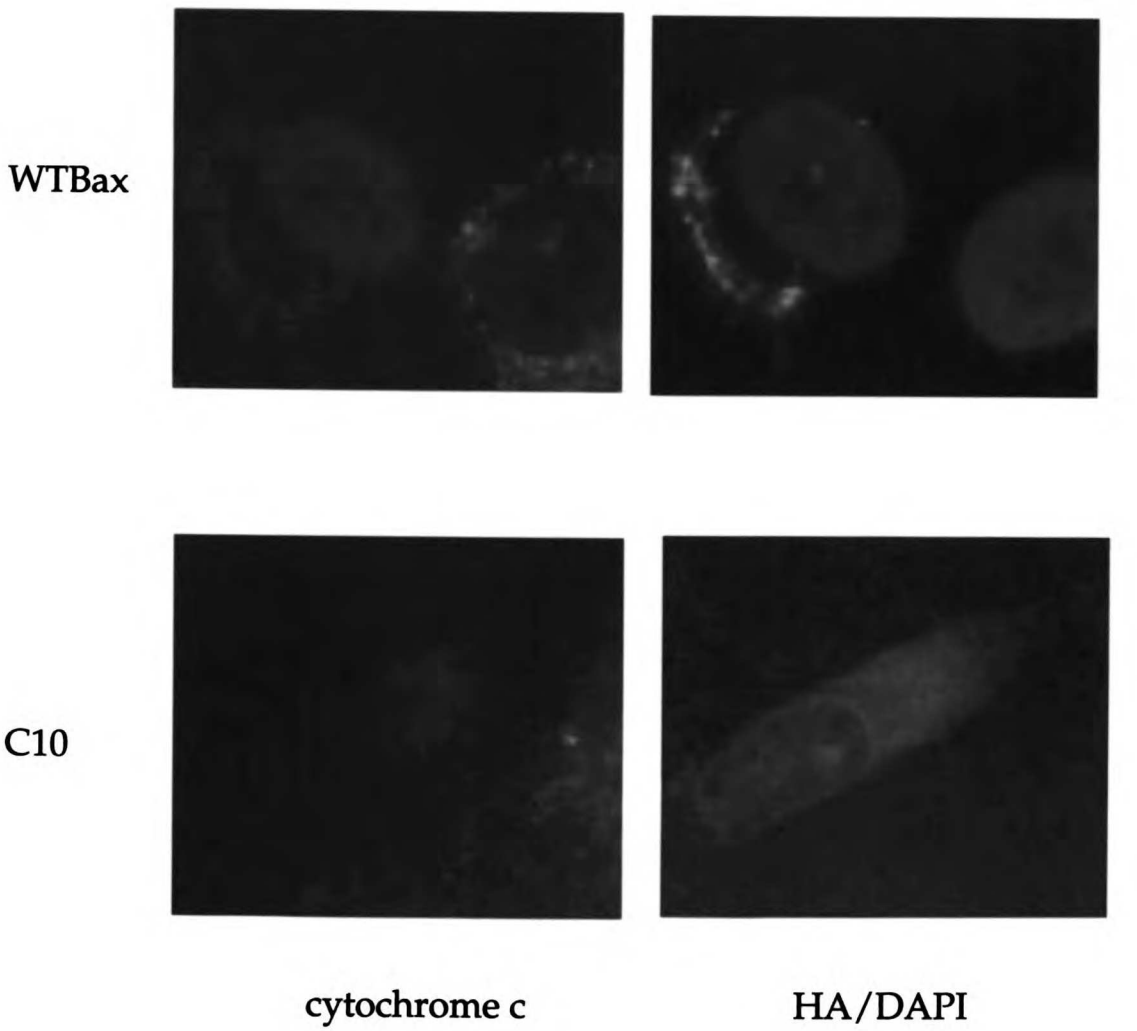
I then assayed which of these mutants could synergize with oligomycin to induce caspase-independent death (Fig. 18C). Each construct was transiently transfected into GM701 cells along with the β-gal reporter; 6 hr later, zVAD-fmk was added to the culture medium either with or without oligomycin, and apoptosis was scored morphologically at 30 hr after transfection. The background level of apoptosis was relatively high (11-15% of cells) in these studies, presumably owing to the transient transfection itself and to the delayed addition of zVAD-fmk; this background apoptosis was reduced (to 8%) in the cells receiving Bcl-2. Importantly, under these conditions, the addition of oligomycin significantly increased the rate of apoptosis induced by Bax and by C10, but not by Bcl-2. Of the remaining chimeras, SW-3 and SW-4 were likewise able to synergize with oligomycin to increase apoptosis, but SW-1 and SW-2 did not. This suggested that residues critical for this synergy are located between positions 61 and 68 of Bax, a region that overlaps the BH3 domain (residues 63-71). Together, these results indicate that an intact Bax BH3 domain is critical for activating caspase-independent death in cooperation with oligomycin.

I had previously characterized two mitochondrial events, cyto c release and perinuclear relocalization, that occurred in I-Bax cells upon induction of Bax

Figure 19. Sequences in or around the Bax BH3 domain are necessary to induce mitochondrial clumping. The indicated HA-tagged constructs were transfected transiently into GM701 cells, treated with zVAD-fmk 6 hr later, and then examined 24 hr post-transfection. Each pair of micrographs depicts a single field showing cyto c (at left) or HA and nuclear staining (at right). (**A**) WTBax and C10. (**B**) SW-1 and SW-2. (**C**) SW-3 and SW-4. (**D**) C10 Upper panel shows HA and nuclear staining, lower panel shows the same cell stained with Mitotracker Green.

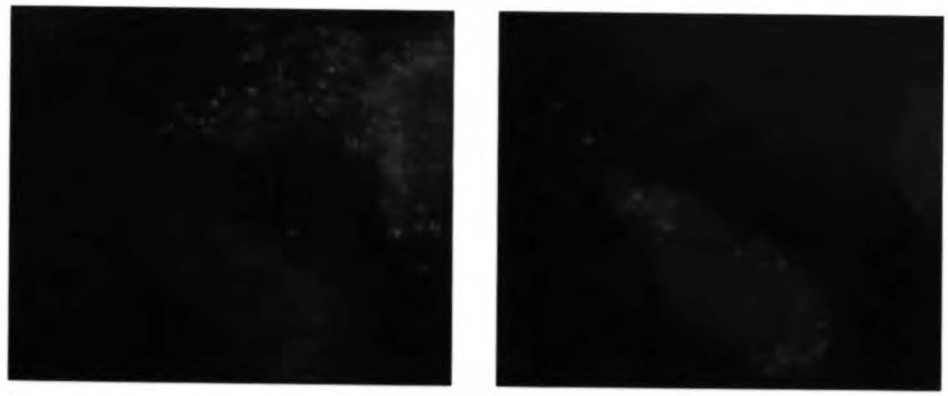
Figure 19

A

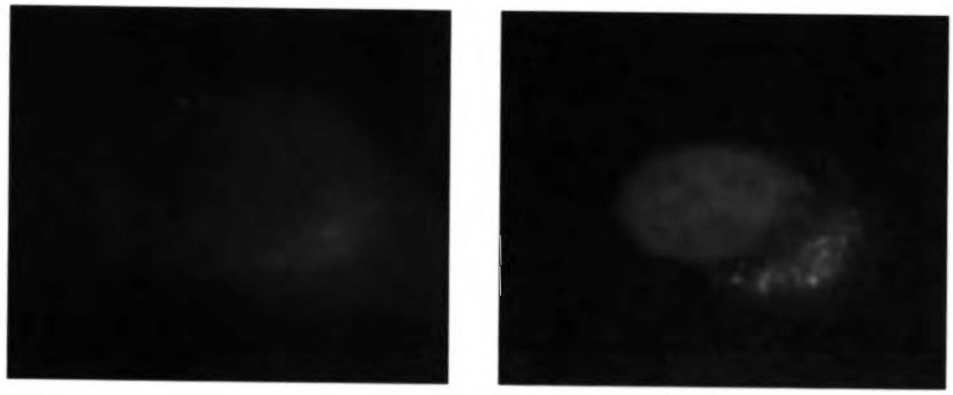


B

SW-1

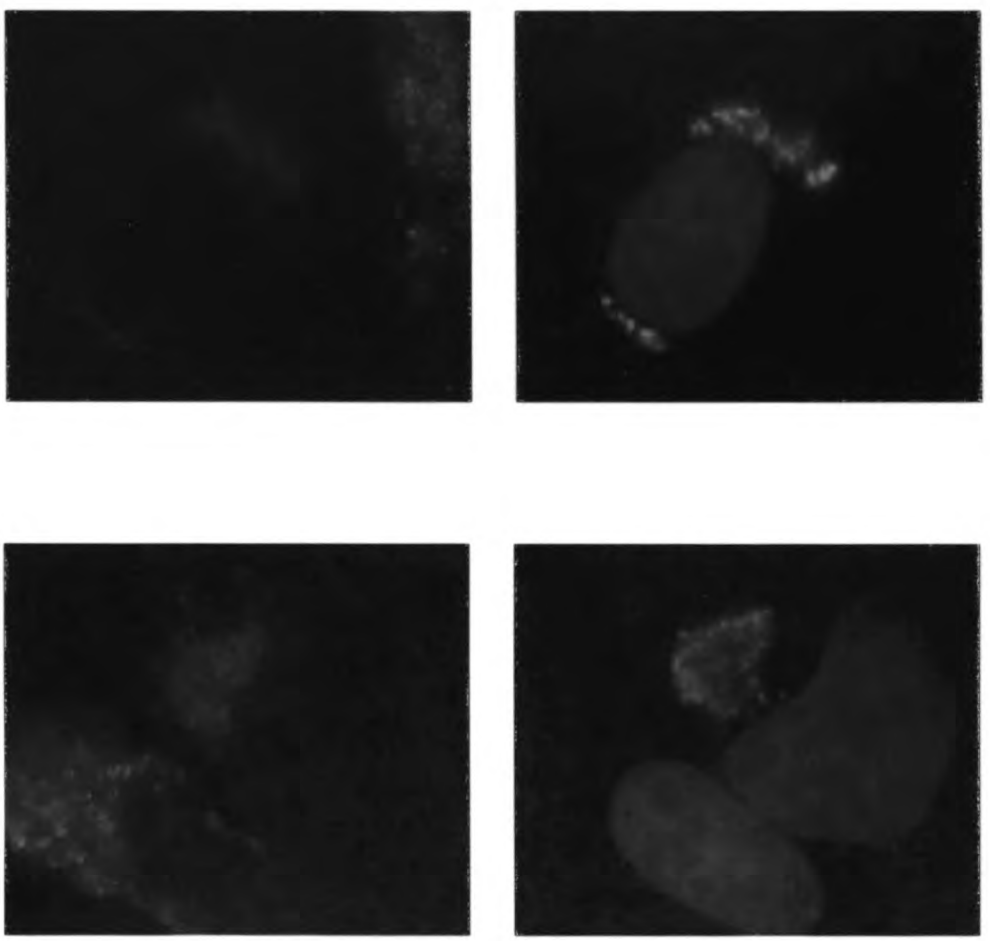


SW-2



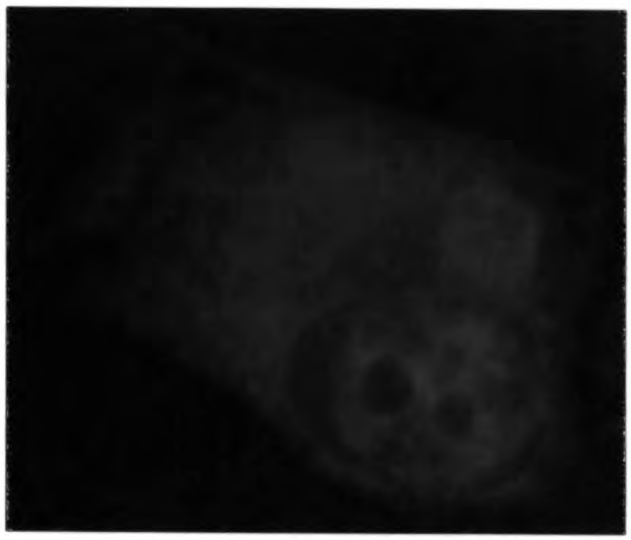
cytochrome c HA/DAPI

C

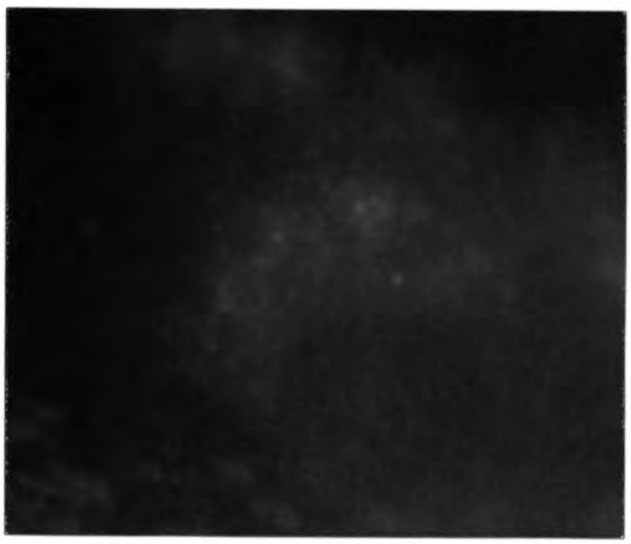


D

C10



HA/DAPI



MitoTracker Green

and that were not affected by the presence of caspase inhibitors. I performed similar immunofluorescence studies in GM701 cells that were transiently transfected with the various Bax/Bcl-2 chimeras to determine if these previous mitochondrial events and oligomycin sensitivity could be separated structurally within Bax. Transfection with WTBax induces an identical pattern as that seen in induced I-Bax cells (Fig. 19A). Chimeras SW1 through SW4 also each associated with mitochondria and induced cyto c release, as judged by a lack of costaining with the cyto c antibody (Fig. 19B and 19C). SW1 and SW2 each lack all or part of the Bax BH3 domain, and so these findings confirm earlier evidence that this domain is not required for mitochondrial localization. By contrast, the C10 protein showed a distribution resembling that of Bcl-2, which associates with diverse cytoplasmic membranes, including the outer nuclear envelope and mitochondria (Fig. 19A). Despite localizing to diverse membranes, C10 was still able to induce clumping as judged by Mitotracker Green staining (Fig. 19D). Interestingly, unlike the other chimeras, SW1 clearly failed to cause clumping of mitochondria (Fig. 19B), suggesting that Bax residues at positions 68-76 might be required for this effect.

I also tested three missense mutants of Bax, each of which contained alanines in place of two consecutive residues within the BH3 domain (Fig. 20A). When transiently expressed in GM701 cells, all three mutants induced apoptosis at least as efficiently as wild-type Bax (Chapter two). Moreover, all three associated with mitochondria and induced both clumping (Fig. 20B) and cyto c release. However, only one mutant was capable of synergizing with oligomycin to induce apoptosis in the presence of zVAD-fmk (Fig. 20A). As the latter

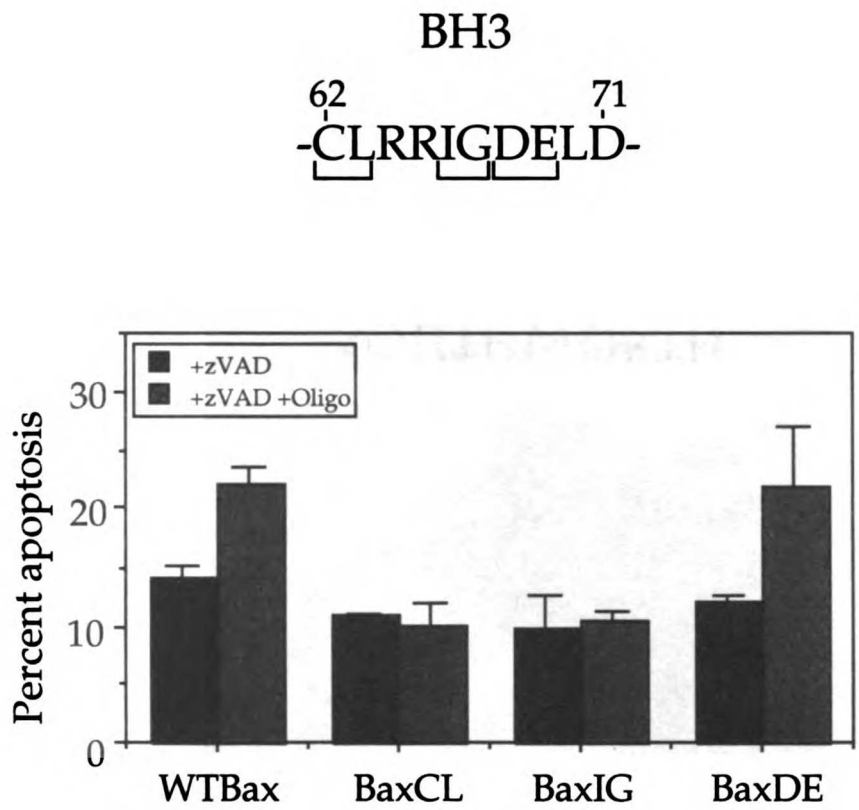
Figure 20. Bax sequences required for caspase-independent killing are distinct from those mediating mitochondrial relocalization.

(**A**) Effect of BH3 point mutations on caspase-independent killing in synergy with oligomycin. Sequence of the wild-type Bax BH3 region is shown at top; brackets indicate three pairs of residues that were replaced by paired alanines to create the three mutants. Below, the apoptogenic activity of each mutant is assayed in GM701 cells treated with zVAD-fmk with or without oligomycin, as in Fig. 5C.

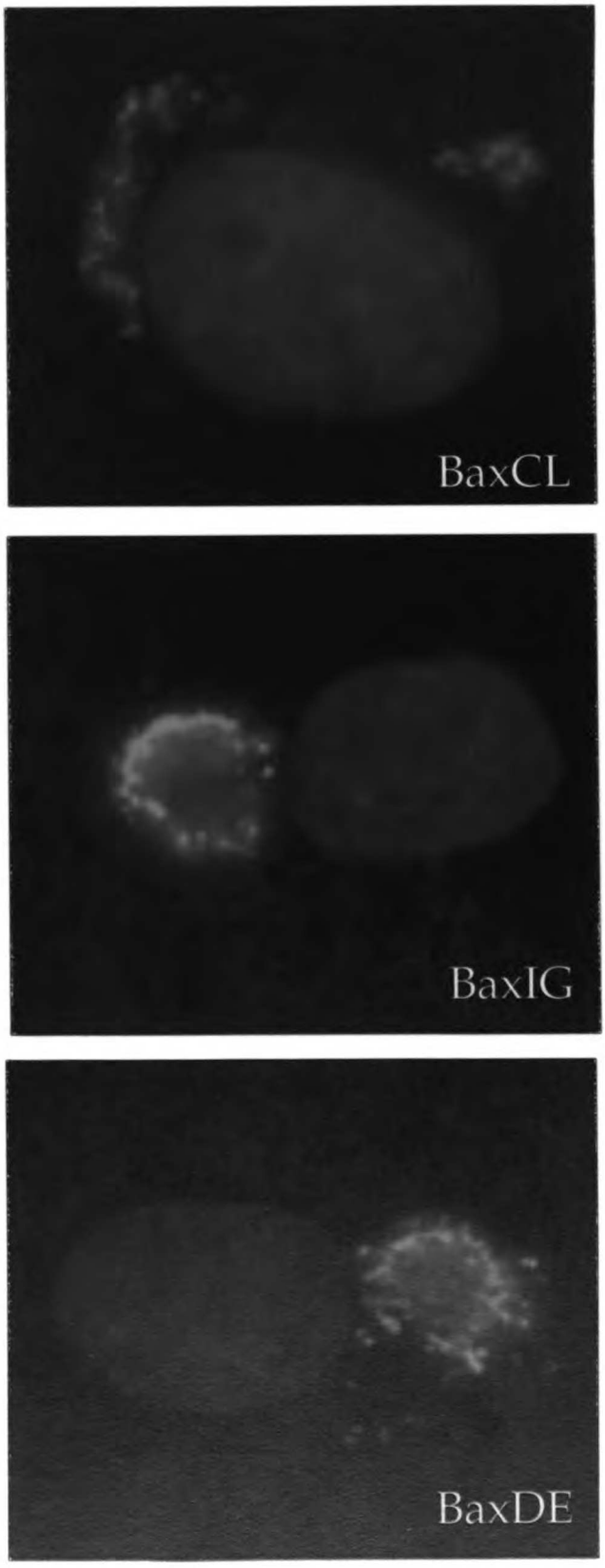
(**B**) Fluorescence micrographs of representative individual cells transiently expressing the indicated Bax mutants in the presence of zVAD-fmk, and stained for HABax (red) and nuclear DNA (blue) as described for Fig. 6.

Figure 20

A


BH3
62
71
-CLRRIGDELD-
+zVAD
+zVAD +Oligo
Percent apoptosis
30
20
10
0
WTBax
BaxCL
BaxIG
BaxDE

B



mutation (BaxDE) involved two residues that are each specifically required for the BH3-mediated dimerization of Bax with Bcl-2 (95), this implies that no such interaction is needed to activate caspase-independent death.

CONCLUSIONS

I had previously characterized a stably transfected cell line, derived from human GM701 fibroblasts, that inducibly expresses the proapoptotic protein Bax. When Bax is induced, these cells undergo a classic apoptotic response that evolves over a period of 3-5 days and can be completely inhibited by pharmacologically blocking caspase activation. Knowing Bax could affect the mitochondria in a caspase independent manner, I tested the synergistic effects between two mitochondrial toxins and Bax in the induction of apoptosis. Both toxins augment Bax induced apoptosis, but interestingly, I find that treatment with oligomycin efficiently triggers death of the Bax-expressing cells even when caspases are inhibited. Cells dying under the latter conditions exhibit several of the defining features of apoptosis, including surface annexin V binding, cytoplasmic shrinkage, and chromatin condensation. These findings verify the existence of a robust, caspase-independent pathway of apoptotic death, and provide an experimental model system in which to study this pathway in isolation. They also imply that a key element of this pathway emanates from mitochondria.

Our data demonstrate that the BH3 domain in Bax is strictly required for its ability to activate caspase-independent death in cooperation with oligomycin. This is in contrast to other properties of Bax, such as mitochondrial localization

and the induction of cyto c release, which do not require an intact BH3 region. Bax's role in activating the second pathway may thus reflect its ability to lower $\Delta\psi_m$, which is known to be BH3-dependent (104), or to form ion channels in cellular membranes (73,88). In our cells, induction of wild-type Bax causes mitochondria to aggregate in a perinuclear region of the cytoplasm; our mutational analysis maps this clumping response to residues in, or immediately downstream of, the BH3 domain. Li et al. (109) have previously noted mitochondrial clumping during the apoptotic response to tBID, another death agonist that contains a BH3 domain. The mechanism of this BH3-dependent clumping is unclear: as similar clumping can also be produced by genetic defects in microtubules (122), it may result from selective detachment of mitochondria away from the microtubular network, or from destruction of microtubules themselves in apoptotic cells. Our data indicate that Bax-induced mitochondrial clumping can occur when caspases are inhibited, but they do not reveal whether such clumping contributes to caspase-independent killing. Significantly, however, I find that another known function of the Bax BH3 domain -- the ability to associate physically with Bcl-2 -- is probably not necessary for activation of this alternative pathway: although previous studies have shown that replacing Bax BH3 residues 68 or 69 individually with alanine prevents binding to Bcl-2 *in vitro*, I find that mutating both these residues simultaneously does not prevent the caspase-independent response (Fig. 20A).

Oligomycin acts on the F_1F_0-ATPase of the mitochondrial inner membrane, which utilizes the energy stored in the transmembrane proton gradient to convert ADP to ATP. By blocking this enzyme, oligomycin prevents

dissipation of the proton gradient and causes mitochondrial hyperpolarization. A functional linkage between Bax and F_1F_0-ATPase was previously suggested on genetic grounds based on a study of Bax-induced killing in yeast (112), but its biochemical basis remains uncertain. Recent studies suggest that Bax binds and inhibits the mitochondrial ATP/ADP antiporter, which pumps ATP out of the organelle in exchange for ADP (111). It may be that, by blocking import of ADP, Bax reduces the availability of this F_1F_0-ATPase substrate, and so enhances vulnerability to oligomycin. Consistent with this model, Bclxl, an antagonist of Bax, prevents oligomycin-induced hyperpolarization of mitochondria (91). Such coupling could account for the synergy I observe, provided that mechanisms are available through which the resulting mitochondrial dysfunction could trigger apoptotic, rather than necrotic, cell death. In this regard, Susin et al. (132) have recently reported purification of apoptosis inducing factor (AIF), a 57-kilodalton flavoprotein that normally resides in the mitochondrial intermembranous space but can be released to the cytoplasm in response to apoptotic stimuli. Once released, AIF potently induces chromatin condensation, large-scale chromosomal fragmentation, loss of $\Delta\psi_m$, and exposure of surface phosphatidylserine; most significantly, these activities are all resistant to inhibition by zVAD-fmk. The effects of AIF, which closely mirror those I observed in dying I-Bax cells, illustrate that many of the classic features of apoptosis can occur without caspase activation. It remains to be determined whether AIF itself, or other mitochondrially-derived effectors, can fully account for caspase-independent cell killing by oligomycin and Bax, and how such effectors lead to the terminal manifestations of apoptotic death.

CHAPTER FIVE

BAX CONTAINS MULTIPLE DOMAINS WHICH INDUCE DISTINCT PROAPOPTOTIC EVENTS

Apoptosis has been studied for several decades, and yet continues to be a mysterious and poorly understood process. Simplistically, it is the process by which an innate cellular suicide program is activated after integration of positive and negative survival signals. But detailed study of the components of this program continually uncovers multiple homologues of each participating protein, each of which appears to function in a slightly different manner to regulate apoptotic outcomes. Bax was first believed to act only as a negative inhibitor of the protective functions of Bcl-2 and Bclxl. The discovery of its apoptosis inducing activity shifted the paradigm so that Bax was believed to be the activator of apoptosis, with Bcl-2 and Bclxl functioning only to inhibit this activity. This model has now been shown to be too simplistic. The current hypothesis is that most members of the Bcl-2 family have an intrinsic ability to activate or inhibit apoptosis, and that it is the integration of their various signals that determines if apoptosis ultimately becomes activated (87, 118). My thesis research has focused on determining the structural features within Bax that are critical for its apoptosis inducing activity, as well as analyzing the downstream events of Bax induced apoptosis using an inducible system that was established in GM701 fibroblasts.

Previous studies had suggested that the apoptosis-activating domain of Bax could be localized to approximately 23 amino acids. My research was begun

with the hypothesis that delineation of the critical amino acids within this region would facilitate the identification of downstream regulators. Point mutagenesis of this killing domain demonstrated that apoptosis inducing activity could be reduced to approximately half of the wild type levels, but no complete loss-of-function mutation was identified. Replacement of the entire domain with the homologous region of Bcl-2 also resulted in a protein that was capable of inducing approximately 50% of wild type induced apoptosis. Thus, although this 23 amino acid domain is capable of conferring killing activity onto Bcl-2, if does not appear to account for all of the killing activity in Bax. The fact that deleting the BH3X region abolishes Bax activity probably reflects not only the effects of the deletion itself but also concomitant changes in the structure of adjacent regions that are required for BH3 independent killing. Whether the latter activity maps to a single domain or to multiple separate domains outside of the BH3 remains to be determined.

I have established a model system in which to study the downstream events of Bax induced apoptosis in GM701 fibroblasts. Using these cells, I have proved that the following events occur after induction of Bax: Bax localizes to mitochondria, cytochrome c is released, and mitochondria move from a broad cytosolic distribution to a clustered location around the nucleus. The recently defined ability of cyto c to interact with Apaf-1 and thereby activate caspase- 9 suggests that Bax induced release of cyto c is sufficient to induce apoptosis and explains most of Bax's killing activity. Consistent with this hypothesis, I find that addition of the pan-caspase inhibitor, zVAD-fmk, can prevent Bax induced cell death for over 72 hours.

I have demonstrated that Bax/Bcl-2 chimeras, which are missing the defined BAX BH3X domain, also localize to the mitochondria and induce the release of cytochrome c, thus explaining their retention of apoptosis inducing ability. I have also shown that a reciprocal swapping mutant, C10, can induce very similar effects, even though its overall cellular distribution is more similar to Bcl-2 than to Bax. C10 and SW-1 are made up of reciprocal domains, and yet each retains the ability to induce cyto c release, suggesting there are two domains within Bax that are capable of inducing this mitochondrial effect. Another explanation is that the Bcl-2 family of proteins avidly forms homo- and heterodimeric interactions with each other, and these chimeras may retain ability to interact with and activate endogenous Bax, or to form inactivating heterodimers with Bcl-2, thus accounting for their overlapping functions. Relevant to this hypothesis is an experiment where artificial dimerization of Bax through an exogenous dimerization domain caused mitochondrial dysfunction without induction of cytochrome c release (102). Unfortunately, the techniques that are utilized to detect dimerization have recently been questioned by the fact that addition of detergents to lysis buffers can create artificial dimerization conditions and so cause misleading results (77). For this reason, no interaction studies were undertaken in my research, but it will be interesting to determine if there is a functional relevance to dimerization among these chimeras once the techniques have been improved.

The unknown mechanism by which cyto c is released from mitochondria is a central question of Bax activity. I have shown that zVAD-fmk blocks the opening of the megachannel associated with apoptosis, and so Bax must induce a

subtler change to the mitochondria that allows for release of this critical signaling molecule. Cyto c is just small enough that it may be able to pass through the constitutive pores of the outer mitochondrial membrane if it is released from the electron transport complex. Usually the pores of the outer membrane do not allow passage of proteins over 5 kD through, but perhaps an apoptotic signal could alter their specificity so that cyto c could pass through. Bax could potentially inhibit the electron transport chain and also induce a change in the outer pore, thus causing the release of cyto c from this complex and allowing it to escape to the cytosol. Bid is another Bcl-2 family member that activates apoptosis. During normal homeostasis, it is present within the cell but is negatively regulated by an N-terminal prodomain, a system that is reminiscent of caspase regulation. Removal of this inhibitory domain by caspase cleavage causes truncated Bid (tBid) to translocate to the mitochondria and release cyto c (109,110). The fact that activated caspases further activate apoptosis by triggering mitochondrial derangement through tBid suggests that activation of the caspases may not be sufficient to fully activate cell death. Also, cyto c is thought to function solely as a signal for the activation of caspases, and it would seem redundant for tBid to only form a cyto c selective pore. A more likely interpretation of tBid, and therefore Bax function is that other constituents of the mitochondria besides cyto c (such as AIF) are necessary for the completion of apoptosis, and that tBid and Bax induce a pronounced change on the mitochondria that releases several factors at once. It will be interestingly to learn what these factors are and if they are all released by each activator, or if, as seems to be the case for the other apoptotic regulatory pathways, there is a substrate

specificity associated with each Bcl-2 family member that gives rise to the functional redundancy in this family.

An exciting function of Bax that I have characterized is its ability to synergize with the F_1F_0-ATPase inhibitory toxin, oligomycin, to induce caspase independent cell death. Bax and the F_1F_0-ATPase had previously been shown to have a functional linkage through the use of a genetic screen in yeast, supporting the relevance of this apoptotic effect. Using our chimeric proteins and point mutants, I have determined that certain amino acids within the BH3 domain of Bax are required for this function. These point mutants were still capable of releasing cyto c and inducing the relocalization of mitochondria, suggesting that these mutations did not induce pronounced structural changes in Bax, but perhaps instead abolished a specific protein-protein interaction site. It will be exciting to determine if there is an unknown protein involved in activating this pathway, and if other proapoptotic regulators, such as tBid and Bik, also interact with this protein. A significant difference I have described between our inducible cell line and one created in Jurkat cells is the sensitivity to caspase inhibition. One mechanism for this difference could be if this unknown protein is regulated in a tissue specific manner, causing T cells to be more sensitive to caspase independent cell death than fibroblasts. It also remains to be determined what occurs after Bax interacts with this protein that allows apoptosis to proceed without caspases. One likely hypothesis is that AIF is released through this interaction. AIF induces apoptotic changes in the presence of zVAD-fmk that are very similar to the ones observed during Bax/oligomycin induced caspase independent death (132). Unfortunately, I was not able to test for the release of

AIF, and so the sequence of release of this key regulatory protein remains to be determined.

The studies described herein have all used overexpression of Bax to determine its function. A key question to be answered is how this applies to the endogenous regulation of Bax and other apoptotic activators. Upon induction of apoptosis by the withdrawal of Tet, HABax appeared to localize predominately to mitochondria, suggesting this to be the principal site of Bax activity. One caveat to these findings is the fact that under normal growth conditions (i.e., with Tet), the transgenic Bax was compartmentalized differently than endogenous Bax, perhaps because the epitope tag interfered in some manner with an endogenous regulator. Placement of an epitope tag at a different site or the development of more informative antibodies against Bax itself will help to answer the question of whether Bax functions at multiple membranes during apoptosis, or if the mitochondrion is truly its only site of action. Bcl-2 localizes to diverse membranes in the cell, and if the mitochondria were the only important site, this would seem to be a very inefficient use of Bcl-2. Bcl-2's function has been proposed to include a direct binding interaction with the cytosolic protein Apaf-1, an interaction that causes inhibition of caspase activation (105,115). If Bax's major activity is the release of cyto c, it would seem necessary that Bax also inactivate this function of Bcl-2, possibly via inactivating heterodimers, so that cyto c could activate Apaf-1. It should be possible to test this hypothesis by making dimerization-deficient mutants of the SW-1 chimera.

The biochemical data from GM701 cells suggest that endogenous Bax is already localized to membranes, and yet these cells are not continuously

undergoing a higher than normal rate of cell death. Goping *et al* have also observed Bax in the membrane fraction of cells before and after an apoptotic stimulus was given (100). They further determined that the membrane fraction of Bax in apoptotic cells is more resistant to alkali stripping, and postulate that Bax can have two conformations on membranes. The first conformation is only loosely associated with membranes, while the second has inserted into the membrane. Interestingly, they also describe an N-terminal inhibitory signal that prevents Bax insertion in mitochondrial membranes, and present evidence that this inhibition is due to an unidentified cytosolic protein. It will be interesting to search for similar membrane conformations in GM701 cells, as well as to determine if HABax adopts alternative conformations even with the exogenous N-terminal epitope tag. If these variations in membrane targeting are observed in both cell types, the pronounced differences between Bax conformations in I-Bax cells may be useful in elucidating this membrane regulatory system.

The research presented in this thesis further defines the complex regulation of apoptosis. The proteins in the Bcl-2 family have forced scientists to examine new paradigms of how molecules can be structurally homologous, and yet have opposing functions, or even to exert several conflicting activities at once. Bcl-2 had previously been described as functioning at multiple points along the apoptotic pathway to inhibit cell death. This research demonstrates that Bax also contains several apoptosis inducing effects, each of which potentially counters a protective effect of Bcl-2. Use of the chimeric proteins and the I-Bax cell line should aid in elucidation of these proapoptotic effects, as well as expose new regulatory mechanisms by which the cell controls the amazing

REFERENCES

1. Iversen, O. H. 1967. Kinetics of cellular proliferation and cell loss in human carcinomas. A discussion of methods available for in vivo studies. Eur J Cancer. 3:389-94.

2. Kerr, J. F., and J. Searle. 1972. A mode of cell loss in malignant neoplasms. J Pathol. 106:xi.

3. Kerr, J. F., and J. Searle. 1972. A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain numerous mitotic figures. J Pathol. 107:41-4.

4. Kerr, J. F., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 26:239-57.

5. Wyllie, A. H., J. F. Kerr, and A. R. Currie. 1973. Cell death in the normal neonatal rat adrenal cortex. J Pathol. 111:255-61.

6. Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. 284:555-6.

7. Tsujimoto, Y., and C. M. Croce. 1986. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci U S A. 83:5214-8.

8. Vaux, D. L., S. Cory, and J. M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 335:440-2.

9. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley. 1992. Mice deficient for p53 are

developmentally normal but susceptible to spontaneous tumours. Nature. 356:215-21.

10. Hengartner, M. O., R. E. Ellis, and H. R. Horvitz. 1992. Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature. 356:494-9.

11. Vaux, D. L., I. L. Weissman, and S. K. Kim. 1992. Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science. 258:1955-7.

12. Yuan, J., and H. R. Horvitz. 1992. The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. Development. 116:309-20.

13. Jacobson, M. D., J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. C. Raff. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature. 361:365-9.

14. Kane, D. J., T. A. Sarafian, R. Anton, H. Hahn, E. B. Gralla, J. S. Valentine, T. Ord, and D. E. Bredesen. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science. 262:1274-7.

15. Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig. 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A. 90:3516-20.

16. Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 75:653-60.

17. Nakayama, K., K. Nakayama, I. Negishi, K. Kuida, Y. Shinkai, M. C. Louie, L. E. Fields, P. J. Lucas, V. Stewart, F. W. Alt, and et al. 1993. Disappearance

of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science. 261:1584-8.

18. Oltvai, Z. N., C. L. Milliman, and S. J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 74:609-19.

19. Veis, D. J., C. M. Sorenson, J. R. Shutter, and S. J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 75:229-40.

20. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. Watson. 1994. Energy Conversion: Mitochondria and Chloroplasts. In Molecular Biology of the Cell. Garland Publisher, Inc., New York & London. p. 652-720.

21. Albrecht, H., J. Tschopp, and C. V. Jongeneel. 1994. Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-kappa B by TNF. FEBS Lett. 351:45-8.

22. Clem, R. J., and L. K. Miller. 1994. Control of programmed cell death by the baculovirus genes p35 and iap. Mol Cell Biol. 14:5212-22.

23. Hengartner, M. O., and H. R. Horvitz. 1994. C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell. 76:665-76.

24. Hengartner, M. O., and H. R. Horvitz. 1994. Programmed cell death in Caenorhabditis elegans. Curr Opin Genet Dev. 4:581-6.

25. Krajewski, S., M. Krajewska, A. Shabaik, T. Miyashita, H. G. Wang, and J. C. Reed. 1994. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol. 145:1323-36.

26. Ramani, P., and Q. L. Lu. 1994. Expression of bcl-2 gene product in neuroblastoma. J Pathol. 172:273-8.

27. Sarafian, T. A., and D. E. Bredesen. 1994. Is apoptosis mediated by reactive oxygen species? Free Radic Res. 21:1-8.

28. Yin, X. M., Z. N. Oltvai, and S. J. Korsmeyer. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature. 369:321-3.

29. Boyd, J. M., G. J. Gallo, B. Elangovan, A. B. Houghton, S. Malstrom, B. J. Avery, R. G. Ebb, T. Subramanian, T. Chittenden, R. J. Lutz, and et al. 1995. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene. 11:1921-8.

30. Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, and et al. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. Science. 269:1885-8.

31. Castedo, M., A. Macho, N. Zamzami, T. Hirsch, P. Marchetti, J. Uriel, and G. Kroemer. 1995. Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo. Eur J Immunol. 25:3277-84.

32. Chao, D. T., G. P. Linette, L. H. Boise, L. S. White, C. B. Thompson, and S. J. Korsmeyer. 1995. Bcl-XL and Bcl-2 repress a common pathway of cell death. J Exp Med. 182:821-8.

33. Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain

of Fas and initiates apoptosis. Cell. 81:505-12.

34. Chittenden, T., C. Flemington, A. B. Houghton, R. G. Ebb, G. J. Gallo, B. Elangovan, G. Chinnadurai, and R. J. Lutz. 1995. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. Embo J. 14:5589-96.

35. Farrow, S. N., J. H. White, I. Martinou, T. Raven, K. T. Pun, C. J. Grinham, J. C. Martinou, and R. Brown. 1995. Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature. 374:731-3.

36. Fernandes-Alnemri, T., G. Litwack, and E. S. Alnemri. 1995. Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res. 55:2737-42.

37. Haas-Kogan, D. A., S. C. Kogan, D. Levi, P. Dazin, A. T'Ang, Y. K. Fung, and M. A. Israel. 1995. Inhibition of apoptosis by the retinoblastoma gene product. Embo J. 14:461-72.

38. Hay, B. A., D. A. Wassarman, and G. M. Rubin. 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell. 83:1253-62.

39. Knudson, C. M., K. S. Tung, W. G. Tourtellotte, G. A. Brown, and S. J. Korsmeyer. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science. 270:96-9.

40. Korsmeyer, S. J., X. M. Yin, Z. N. Oltvai, D. J. Veis-Novack, and G. P. Linette. 1995. Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. Biochim Biophys Acta. 1271:63-6.

41. Krajewski, S., C. Blomqvist, K. Franssila, M. Krajewska, V. M. Wasenius, E.

Niskanen, S. Nordling, and J. C. Reed. 1995. Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res. 55:4471-8.

42. Miyashita, T., and J. C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 80:293-9.

43. Motoyama, N., F. Wang, K. A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, and et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science. 267:1506-10.

44. Shimizu, S., Y. Eguchi, H. Kosaka, W. Kamiike, H. Matsuda, and Y. Tsujimoto. 1995. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature. 374:811-3.

45. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 184:39-51.

46. Xue, D., and H. R. Horvitz. 1995. Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature. 377:248-51.

47. Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson, and S. J. Korsmeyer. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. 80:285-91.

48. Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A.

Thornberry, W. W. Wong, and J. Yuan. 1996. Human ICE/CED-3 protease nomenclature. Cell. 87:171.

49. Boise, L. H., and C. B. Thompson. 1996. Hierarchical control of lymphocyte survival. Science. 274:67-8.

50. Cheng, E. H., B. Levine, L. H. Boise, C. B. Thompson, and J. M. Hardwick. 1996. Bax-independent inhibition of apoptosis by Bcl-XL. Nature. 379:554-6.

51. Duan, H., K. Orth, A. M. Chinnaiyan, G. G. Poirier, C. J. Froelich, W. W. He, and V. M. Dixit. 1996. ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. J Biol Chem. 271:16720-4.

52. Duckett, C. S., V. E. Nava, R. W. Gedrich, R. J. Clem, J. L. Van Dongen, M. C. Gilfillan, H. Shiels, J. M. Hardwick, and C. B. Thompson. 1996. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. Embo J. 15:2685-94.

53. Froelich, C. J., K. Orth, J. Turbov, P. Seth, R. Gottlieb, B. Babior, G. M. Shah, R. C. Bleackley, V. M. Dixit, and W. Hanna. 1996. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem. 271:29073-9.

54. Hawkins, C. J., A. G. Uren, G. Hacker, R. L. Medcalf, and D. L. Vaux. 1996. Inhibition of interleukin 1 beta-converting enzyme-mediated apoptosis of mammalian cells by baculovirus IAP. Proc Natl Acad Sci U S A. 93:13786-90.

55. Hunter, J. J., and T. G. Parslow. 1996. A peptide sequence from Bax that converts Bcl-2 into an activator of apoptosis. J Biol Chem. 271:8521-4.

56. Kitada, S., S. Krajewski, T. Miyashita, M. Krajewska, and J. C. Reed. 1996. Gamma-radiation induces upregulation of Bax protein and apoptosis in radiosensitive cells in vivo. Oncogene. 12:187-92.

57. Lippke, J. A., Y. Gu, C. Sarnecki, P. R. Caron, and M. S. Su. 1996. Identification and characterization of CPP32/Mch2 homolog 1, a novel cysteine protease similar to CPP32. J Biol Chem. 271:1825-8.

58. Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 86:147-57.

59. Marchetti, P., S. A. Susin, D. Decaudin, S. Gamen, M. Castedo, T. Hirsch, N. Zamzami, J. Naval, A. Senik, and G. Kroemer. 1996. Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. Cancer Res. 56:2033-8.

60. Middleton, G., G. Nunez, and A. M. Davies. 1996. Bax promotes neuronal survival and antagonises the survival effects of neurotrophic factors. Development. 122:695-701.

61. Muchmore, S. W., M. Sattler, H. Liang, R. P. Meadows, J. E. Harlan, H. S. Yoon, D. Nettesheim, B. S. Chang, C. B. Thompson, S. L. Wong, S. L. Ng, and S. W. Fesik. 1996. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature. 381:335-41.

62. Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer,

M. E. Peter, and V. M. Dixit. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell. 85:817-27.

63. Simonian, P. L., D. A. Grillot, D. W. Andrews, B. Leber, and G. Nunez. 1996. Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death. J Biol Chem. 271:32073-7.

64. Susin, S. A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer. 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J Exp Med. 184:1331-41.

65. Takahashi, A., E. S. Alnemri, Y. A. Lazebnik, T. Fernandes-Alnemri, G. Litwack, R. D. Moir, R. D. Goldman, G. G. Poirier, S. H. Kaufmann, and W. C. Earnshaw. 1996. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc Natl Acad Sci U S A. 93:8395-400.

66. Uren, A. G., M. Pakusch, C. J. Hawkins, K. L. Puls, and D. L. Vaux. 1996. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci U S A. 93:4974-8.

67. Wang, K., X. M. Yin, D. T. Chao, C. L. Milliman, and S. J. Korsmeyer. 1996. BID: a novel BH3 domain-only death agonist. Genes Dev. 10:2859-69.

68. Wang, S., M. Miura, Y. Jung, H. Zhu, V. Gagliardini, L. Shi, A. H. Greenberg, and J. Yuan. 1996. Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an

upstream regulator of ICE. J Biol Chem. 271:20580-7.

69. Xiang, J., D. T. Chao, and S. J. Korsmeyer. 1996. BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. Proc Natl Acad Sci U S A. 93:14559-63.

70. Zamzami, N., P. Marchetti, M. Castedo, T. Hirsch, S. A. Susin, B. Masse, and G. Kroemer. 1996. Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. FEBS Lett. 384:53-7.

71. Zha, H., H. A. Fisk, M. P. Yaffe, N. Mahajan, B. Herman, and J. C. Reed. 1996. Structure-function comparisons of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell Biol. 16:6494-508.

72. Diaz, J. L., T. Oltersdorf, W. Horne, M. McConnell, G. Wilson, S. Weeks, T. Garcia, and L. C. Fritz. 1997. A common binding site mediates heterodimerization and homodimerization of Bcl-2 family members. J Biol Chem. 272:11350-5.

73. Antonsson, B., F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Marinou, L. Bernasconi, A. Bernard, Ml Ml Mermod, G. Mazzei, et al. 1997. Inhibition of Bax channel-forming activity by Bcl-2. Science. 277:370-372.

74. Elangovan, B., and G. Chinnadurai. 1997. Functional dissection of the pro-apoptotic protein Bik. Heterodimerization with anti-apoptosis proteins is insufficient for induction of cell death. J Biol Chem. 272:24494-8.

75. Eliasson, M. J., K. Sampei, A. S. Mandir, P. D. Hurn, R. J. Traystman, J. Bao, A. Pieper, Z. Q. Wang, T. M. Dawson, S. H. Snyder, and V. L. Dawson. 1997. Poly(ADP-ribose) polymerase gene disruption renders mice resistant to

cerebral ischemia. Nat Med. 3:1089-95.

76. Hsu, Y. T., K. G. Wolter, and R. J. Youle. 1997. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A. 94:3668-72.

77. Hsu, Y. T., and R. J. Youle. 1997. Nonionic detergents induce dimerization among members of the Bcl-2 family. J Biol Chem. 272:13829-34.

78. Jurgensmeier, J. M., S. Krajewski, R. C. Armstrong, G. M. Wilson, T. Oltersdorf, L. C. Fritz, J. C. Reed, and S. Ottilie. 1997. Bax- and Bak-induced cell death in the fission yeast Schizosaccharomyces pombe. Mol Biol Cell. 8:325-39.

79. Kim, T. W., W. H. Pettingell, Y. K. Jung, D. M. Kovacs, and R. E. Tanzi. 1997. Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science. 277:373-6.

80. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 275:1132-6.

81. Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski, and L. T. Williams. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. 278:294-8.

82. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 91:479-89.

83. Liu, X., H. Zou, C. Slaughter, and X. Wang. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell. 89:175-84.

84. McCurrach, M. E., T. M. Connor, C. M. Knudson, S. J. Korsmeyer, and S. W. Lowe. 1997. bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc Natl Acad Sci U S A. 94:2345-9.

85. Minn, A. J., P. Velez, S. L. Schendel, H. Liang, S. W. Muchmore, S. W. Fesik, M. Fill, and C. B. Thompson. 1997. Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature. 385:353-7.

86. Reed, J. C. 1997. Cytochrome c: can't live with it--can't live without it. Cell. 91:559-62.

87. Reed, J. C. 1997. Double identity for proteins of the Bcl-2 family. Nature. 387:773-6.

88. Schlesinger, P. H., A. Gross, X. M. Yin, K. Yamamoto, M. Saito, G. Waksman, and S. J. Korsmeyer. 1997. Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci U S A. 94:11357-62.

89. Simonen, M., H. Keller, and J. Heim. 1997. The BH3 domain of Bax is sufficient for interaction of Bax with itself and with other family members and it is required for induction of apoptosis. Eur J Biochem. 249:85-91.

90. Van de Craen, M., I. Van den Brande, W. Declercq, M. Irmler, R. Beyaert, J. Tschopp, W. Fiers, and P. Vandenabeele. 1997. Cleavage of caspase family members by granzyme B: a comparative study in vitro. Eur J Immunol.

27:1296-9.

91. Vander Heiden, M. G., N. S. Chandel, E. K. Williamson, P. T. Schumacker, and C. B. Thompson. 1997. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell. 91:627-37.

92. Villa, P., S. H. Kaufmann, and W. C. Earnshaw. 1997. Caspases and caspase inhibitors. Trends Biochem Sci. 22:388-93.

93. Wolter, K. G., Y. T. Hsu, C. L. Smith, A. Nechushtan, X. G. Xi, and R. J. Youle. 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. 139:1281-92.

94. Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 275:1129-32.

95. Zha, H., and J. C. Reed. 1997. Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J Biol Chem. 272:31482-8.

96. Zou, H., W. J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 90:405-13.

97. Adams, J. M., and S. Cory. 1998. The Bcl-2 protein family: arbiters of cell survival. Science. 281:1322-6.

98. Bossy-Wetzel, E., D. D. Newmeyer, and D. R. Green. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. Embo J. 17:37-49.

99. Eskes, R., B. Antonsson, A. Osen-Sand, S. Montessuit, C. Richter, R. Sadoul, G. Mazzei, A. Nichols, and J. C. Martinou. 1998. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J Cell Biol. 143:217-24.

100. Goping, I. S., A. Gross, J. N. Lavoie, M. Nguyen, R. Jemmerson, K. Roth, S. J. Korsmeyer, and G. C. Shore. 1998. Regulated targeting of BAX to mitochondria. J Cell Biol. 143:207-15.

101. Green, D. R., and J. C. Reed. 1998. Mitochondria and apoptosis. Science. 281:1309-12.

102. Gross, A., J. Jockel, M. C. Wei, and S. J. Korsmeyer. 1998. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. Embo J. 17:3878-85.

103. Hakem, R., A. Hakem, G. S. Duncan, J. T. Henderson, M. Woo, M. S. Soengas, A. Elia, J. L. de la Pompa, D. Kagi, W. Khoo, J. Potter, R. Yoshida, S. A. Kaufman, S. W. Lowe, J. M. Penninger, and T. W. Mak. 1998. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell. 94:339-52.

104. Han, J., D. Modha, and E. White. 1998. Interaction of E1B 19K with Bax is required to block Bax-induced loss of mitochondrial membrane potential and apoptosis. Oncogene. 17:2993-3005.

105. Hu, Y., M. A. Benedict, D. Wu, N. Inohara, and G. Nunez. 1998. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci U S A. 95:4386-91.

106. Jurgensmeier, J. M., Z. Xie, Q. Deveraux, L. Ellerby, D. Bredesen, and J. C.

Reed. 1998. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A. 95:4997-5002.

107. Kelekar, A., and C. B. Thompson. 1998. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. 8:324-30.

108. Kuida, K., T. F. Haydar, C. Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. S. Su, P. Rakic, and R. A. Flavell. 1998. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell. 94:325-37.

109. Li, H., H. Zhu, C. J. Xu, and J. Yuan. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 94:491-501.

110. Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 94:481-90.

111. Marzo, I., C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. Vieira, M. C. Prevost, Z. Xie, S. Matsuyama, J. C. Reed, and G. Kroemer. 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science. 281:2027-31.

112. Matsuyama, S., Q. Xu, J. Velours, and J. C. Reed. 1998. The Mitochondrial F0F1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells. Mol Cell. 1:327-36.

113. Muzio, M., B. R. Stockwell, H. R. Stennicke, G. S. Salvesen, and V. M. Dixit. 1998. An induced proximity model for caspase-8 activation. J Biol Chem. 273:2926-30.

114. Narita, M., S. Shimizu, T. Ito, T. Chittenden, R. J. Lutz, H. Matsuda, and Y.

Tsujimoto. 1998. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A. 95:14681-6.

115. Pan, G., K. O'Rourke, and V. M. Dixit. 1998. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol Chem. 273:5841-5.

116. Pastorino, J. G., S. T. Chen, M. Tafani, J. W. Snyder, and J. L. Farber. 1998. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem. 273:7770-5.

117. Peter, M. E., and P. H. Krammer. 1998. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. Curr Opin Immunol. 10:545-51.

118. Rosse, T., R. Olivier, L. Monney, M. Rager, S. Conus, I. Fellay, B. Jansen, and C. Borner. 1998. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature. 391:496-9.

119. Ruck, A., M. Dolder, T. Wallimann, and D. Brdiczka. 1998. Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. FEBS Lett. 426:97-101.

120. Savitz, S. I., and D. M. Rosenbaum. 1998. Apoptosis in neurological disease. Neurosurgery. 42:555-72; discussion 573-4.

121. Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri, and E. S. Alnemri. 1998. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell. 1:949-57.

122. Tanaka, Y., Y. Kanai, Y. Okada, S. Nonaka, S. Takeda, A. Harada, and N. Hirokawa. 1998. Targeted disruption of mouse conventional kinesin

heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria. Cell. 93:1147-58.

123. Thornberry, N. A., and Y. Lazebnik. 1998. Caspases: enemies within. Science. 281:1312-6.

124. Unplublished results. 1998. .

125. Wang, K., A. Gross, G. Waksman, and S. J. Korsmeyer. 1998. Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. Mol Cell Biol. 18:6083-9.

126. Chou, J. J., H. Li, G. S. Salvesen, J. Yuan, and G. Wagner. 1999. Solution structure of BID, an intracellular amplifier of apoptotic signaling. Cell. 96:615-24.

127. Finucane, D. M., E. Bossy-Wetzel, N. J. Waterhouse, T. G. Cotter, and D. R. Green. 1999. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J Biol Chem. 274:2225-33.

128. Imai, Y., T. Kimura, A. Murakami, N. Yajima, K. Sakamaki, and S. Yonehara. 1999. The CED-4-homologous protein FLASH is invlolved in Fas-mediated activation of caspase-8 during apoptosis. Nature. 398:777-84.

129. McDonnell, J. M., D. Fushman, C. L. Milliman, S. J. Korsmeyer, and D. Cowburn. 1999. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. Cell. 96:625-34.

130. Rathmell, J. C., and C. B. Thompson. 1999. The central effectors of cell death in the immune system. Annual Review of Immunology. 17:781-828.

131. Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, C. Brenner, N. Larochette,

M. C. Prevost, P. M. Alzari, and G. Kroemer. 1999. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med. 189:381-94.

132. Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 397:441-6.

APPENDICES

Bax Nucleic Acid Mutations

Name	Published as	Original Nucleic Acids	Mutated to...
2Bax	WTBax	ATG,(*HA-TACCCTTACGAC-*GTTCCTGACTACGCA), 4- 24 (BglII, AGATCT)25-579	- - -
33Bax		Δ25-48	Deletion
11.215		*100-2 CGA, 121-3, GAG	GCA, GCG
4.170		*127-132, CCTGAG	GCTGCG
9.169		*133-138, CTGACC	CTAAGC
3.178		*142-7, GAGCAG	GCGGCG
18.178		*142-7, GAGCAG +136-50 at 153	GCGGCG AGCTTGGAGCAG CCG
4.179		*148-53, CCGCCC	GCGGCC
10.180		*154-59, CAGGAT	GCGGCT
1.214		*148-59,CCGCCCCAGGAT	GCGGCCGCGGCT
6.181		*163-68, TCCACC	GCCGCC
9.182		*169-74, AAGAAG	GCGGCG
17.155	BaxCL	184-89, TGTCTC	GCTGCC
9.156		175-77,CTG, 196-201, ATTGGA	TTC, GCTGCA
6.156	BaxIG	196-201, ATTGGA	GCTGCG
6.216		*172-74, AAG, 193-95,CGA	GCG, GCA
6.218		*169-171, AAG, 190-92, CGG	GCG, GCG
18.157	BaxDE	202-07, GATGAA	GCTGCA
16.235		202-07, GATGAA	CGGCGA
5.348		211-13, GAC	GCC
		* = also contains CTGACC at positions 133-138	

Bax Amino Acid Mutations

Name	Published as	Original Residues	Mutated to...
2Bax	WTBax	1, (HA- YPYDVPDYA), 2-8, (BglII, RS), 9-190	- - -
33Bax		Δ9-16	Deletion
11.215		34,41 R,E *	A,A
4.170		43,44 P,E *	A,A
9.169		46, T	S
3.178		48,49 E ,Q *	A,A
18.178		48,49 E,Q *	A,A
		+SLEQPP	Insertion at 51
4.179		50,51 P,P *	A,A
10.180		52,53 Q,D *	A,A
1.214		50-53, P,P,Q,D *	A,A,A,A
6.181		55,56 S,T *	A,A
9.182		57,58 K,K *	A,A
17.155	BaxCL	62,63 C,L	A,A
9.156		59, 66,67 L, I,G	F, A,A
6.156	BaxIG	66,67 I,G	A,A
6.216		58,65, K,R *	A,A
6.218		57,64, K, R *	A,A
18.157	BaxDE	68,69, D,E	A,A
16.235		68,69, D,E	R,R
5.348		71, D	A
		*=also contains S -> T mutation at residue 46	

Becton Dickenson FACSort Instrument Settings

Annexin V- FITC (FL1) vs. Propidium Iodide (FL2)

Detectors/Amps

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E-1	5.77	Lin
P2	SSC	230	1.00	Log
P3	FL1	504	1.00	Log
P4	FL2	425	1.00	Log
P5	FL3	264	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin

Threshold:
Parameter: FSC
Value: 52

Compensation:
FL1 - 45.4% FL2
FL2 - 19.9% FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2

$DiOC_6$ (FL1)

Detectors/Amps

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E-1	6.42	Lin
P2	SSC	190	1.00	Log
P3	FL1	313	1.00	Log
P4	FL2	293	1.00	Log
P5	FL3	264	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin

Threshold:
Parameter: FSC
Value: 52

Compensation:
FL1 - 0.0% FL2
FL2 - 0.0% FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2

JC-1 monomer (FL1) vs JC-1 dimer (FL2)

Detectors/Amps

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E-1	5.87	Lin
P2	SSC	252	1.00	Log
P3	FL1	358	1.00	Log
P4	FL2	271	1.00	Log
P5	FL3	264	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin

Threshold:
Parameter: FSC
Value: 52

Compensation:
FL1 - 18.1% FL2
FL2 - 19.9% FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2

Propidium Iodide (FL2)

Detectors/Amps

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E-1	6.20	Lin
P2	SSC	178	1.00	Log
P3	FL1	150	1.00	Log
P4	FL2	279	1.00	Log
P5	FL3	150	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin

Threshold:
Parameter: FSC
Value: 52

Compensation:
FL1 - 0.0% FL2
FL2 - 0.0% FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2