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# The Anti-apoptotic Function of Adenovirus E1B 19kDa Protein

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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## *Abstract*

E1B 19kDa protein (19K), a member of Bcl-2 family of protein, is an adenovirus early gene product. It functions to suppress apoptosis of the host cell caused by viral infection. The exact role of E1B 19kDa in regulating apoptosis in response to many different death stimuli has not been investigated.

We found that hygromycin B, an aminoglycoside antibiotic that is used to establish stable mammalian cell lines, kill cells by apoptosis. Apoptosis induced by Hygromycin B is p53-independent and can be blocked both by E1B 19kDa and Bcl-2, showing that E1B 19kDa, like Bcl-2, may confer protection against apoptosis in a variety of circumstances.

The sequence similarities between E1B 19kDa and Bcl-2 are largely restricted to the BH1 and BH3 domains. Our sequence alignment analysis shows that E1B 19kDa also contains a BH4 region overlapping its BH3 domain. We find that the BH1 domain of E1B 19kDa contributes to its anti-apoptotic function and its ability to interact with Bax. A conserved residue Gly 87 in the BH1 domain of E1B 19kDa that is critical for Bcl-2 and Bax interaction is also important for the E1B 19kDa and Bax interaction. We do not detect an interaction between E1B 19kDa and Bad, suggesting that E1B 19kDa is a structural and functional homolog of Bcl-2 but also has its unique features.

Bap31, a 28kDa polytoptic integral protein of the endoplasmic reticulum, is part of an ER protein complex that also includes Bcl-2/Bcl-xL, procaspase-8, and a CED-4-like protein. E1B 19kDa does not interact with Bap31; nor does it interact with Bcl-2/Bcl-xL. We have made a set of chimeric E1B 19kDa proteins carrying different BH domains from Bcl-2 to test their protein-protein interaction properties. We show that the BH3 domain of Bcl-2, when substituted for the homologous region of E1B 19kDa, confers the properties of interaction with Bap31 and Bcl-xL onto 19K. The subcellular distribution and antiapoptotic function of the 19K/Bcl-2BH3 is very similar to that of wild type 19K. These results show that the BH3 domain of Bcl-2/Bcl-xL has an important role in mediating Bcl-2/Bcl-xL and Bap31 interaction.

## *Résumé*

La protéine E1B-19K, un membre de la famille des protéines apparentées à Bcl-2, est codée par un gène précoce des adénovirus. La fonction de E1B-19K est de bloquer l'apoptose induite chez la cellule hôte lors de l'infection virale. Le rôle précis de E1B-19K dans la régulation de l'apoptose en réponse à d'autres stimuli n'avait pas encore été étudié.

Nous avons observé que l'hygromycine B, un antibiotique aminoglycosidique utilisé pour établir des lignées cellulaires stables, tue les cellules par apoptose. Cette mort cellulaire survient aussi bien en absence qu'en présence de la protéine p53 et elle peut être bloquée par E1B-19K et Bcl-2 démontrant que E1B-19K, comme Bcl-2, peut protéger la cellule contre l'apoptose dans plus d'une situation.

Les similitudes entre les séquences en acides aminés des protéines E1B-19K et Bcl-2 se limitent principalement aux domaines BH1 et BH3. Notre analyse des séquences de ces deux protéines démontre que E1B-19K contient aussi une région BH4, superposée au domaine BH3. Les résultats obtenus démontrent que le domaine BH1 de E1B-19K est essentiel à sa fonction anti-apoptose ainsi qu'à la formation d'un hétérodimère avec la protéine Bax. Le résidu d'acide aminé glycine 87 retrouvé dans le domaine BH1, essentiel pour l'interaction entre Bcl-2 et Bax, s'est avéré être important pour l'interaction entre E1B-19K et Bax. Nous n'avons pas détecté d'interaction entre E1B-19K et Bad ce qui suggère que E1B-19K est un homologue structural et fonctionnel de Bcl-2 mais qu'il possède aussi des caractéristiques qui lui sont propres.

Bap31, une protéine transmembranaire de 28 kDa retrouvée dans le réticulum endoplasmique, est partie intégrante d'un complexe de protéines qui comprend aussi Bcl-2/Bcl-xL, procaspase-8 et une protéine similaire à CED-4. E1B-19K ne se lie pas à Bap31 pas plus qu'elle n'interagit avec Bcl-2/Bcl-xL. Nous avons généré des protéines E1B-19K chimériques comportant différents domaines d'homologie à Bcl-2 (BH) afin de tester leur habileté à se lier à d'autres protéines. Nous montrons que la substitution du domaine BH3 de E1B-19K par celui de Bcl-2, confère à E1B-19K l'habileté à se lier avec Bap31 et Bcl-xL. La distribution subcellulaire et la fonction anti-apoptose de cette protéine chimérique E1B-

19K/Bcl-2BH3 est très semblable à celle de la protéine E1B-19K intacte. Ces résultats démontrent que le domaine BH3 de Bcl-2/Bcl-xL joue un rôle important dans l'interaction entre Bcl-2/Bcl-xL et Bap31.

## *Acknowledgements*

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## *List of Abbreviations*

| | |
|---|---|
| ψ | membrane potential |
| aa | amino acid |
| Ad5 | Adenovirus type 5 |
| ABC | ATP binding cassette |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| AIDS | acquired immunodeficiency syndrome |
| AIF | apoptosis-inducing factor |
| Akt | a serine-threonine kinase also known as PKB |
| ANT | adenine nucleotide translocator |
| Apaf | apoptosis protease activating factor |
| ART | apoptotic regulation of targeting |
| ATM | ataxia-telangiectasia gene |
| BH | Bcl-2 homology |
| bp | base pair |
| BAP31 | BCR associated protein 31 |
| BCR | B cell receptor |
| BI-1 | Bax inhibitor protein-1 |
| BIR | baculovirus IAP repeat |
| BRK | Baby Rat Kidney |
| CAD | caspase-activated DNAse, also known as DFF40 |
| cAMP | cyclic adenosine monophosphate |
| CARD | caspase recruitment domain |
| Caspase | cysteinyl aspartate-specific proteinase |
| CDK | cyclin-dependent kinase |
| cDNA | complementary DNA |
| *ced* | cell death gene |
| CR1 | conserved region 1 |
| CR2 | conserved region 2 |
| CR3 | conserved region 3 |
| CrmA | cytokine response modifier A |
| C-terminal | carboxy terminal |
| CTL | cytotoxic T lymphocyte |
| DD | death domain |
| DED | death effector domain |
| DEVD-fmk | acetyl Asp-Glu-Val-Asp-fluoromethylketone |
| DFF | DNA fragmentation factor |
| DISC | death inducing signalling complex |
| DNA | deoxyribonucleic acid |
| DNA-PK | DNA dependent protein kinase |
| DR3 | death receptor 3 |
| DR4 | death receptor 4 |
| ds | double-stranded |

| | |
|---|---|
| E1A | early region 1A |
| E1B | early region 1B |
| E3 | early region 3 |
| E4 | early region 4 |
| EGF | epidermal growth factor |
| EGF-R | epidermal growth factor receptor |
| ER | endoplasmic reticulum |
| FADD | Fas-associated death domain |
| FAP | Fas-associated phosphatase |
| HIV | human immunodeficiency virus |
| FLICE | FADD-like-ICE/Ced-3-like protease |
| FLIP | FLICE inhibitory protein |
| HPV | human papillomavirus |
| HVS | herpesvirus saimiri |
| IAP | inhibitor of apoptosis protein |
| ICAD | inhibitor of CAD, also known as DFF45 |
| ICE | interleukin-1β-converting enzyme |
| Igh | immunoglobulin heavy chain |
| JNK | c-jun N-terminal kinase |
| IP3R-1 | inositol-1, 4, 5-trisphophate receptor |
| SAPK | stress activated protein kinase |
| kb | kilobase |
| kDa | kilodalton |
| M phase | mitosis phase |
| MAPK | mitogen activated protein kinase |
| MDM2 | murine double minute 2 gene product |
| MEK | MAPK/ERK kinase |
| MEKK | MEK kinase |
| MHC | major histocompatibility complex |
| mRNA | messenger RNA |
| NAIP | the neuronal apoptosis inhibitory protein |
| NGF | nerve growth factor |
| N-terminal | amino terminal |
| nm | nanometer |
| NMR | nuclear magnetic resonance spectroscopy |
| orf | open reading frame |
| PARP | poly(ADP-ribose) polymerase |
| PH | plextrin homology |
| PI3K | phosphatidylinositol-3-kinase |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PKR | protein kinase R |
| poly(A) | polyadenylation |
| PP2A | protein phosphatase 2A |
| PT | permeability transition |
| PTPC | permeability transition pore complex |

| | |
|---|---|
| PtdSer | phospholipid phosphatidylserine |
| RAIDD | RIP-associated ICH-1 homologous protein with death domain |
| Rb | retinoblastoma susceptibility gene product |
| RID | receptor internalization and degradation complex (E3-14.5K/E3-10.4K) |
| RIP | receptor-interacting protein |
| RNA | ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SM | sphingomyelin |
| SMase | Sphingomyelinase |
| S phase | DNA synthesis phase |
| SPP | sphingosine-1-phosphate |
| ss | single stranded |
| TNF | tumour necrosis factor |
| TNFR | tumour necrosis factor receptor |
| TRADD | TNFR-associated death domain |
| XIAP | X-chromosome-linked iap gene product |
| VDAC | voltage-dependent anion channel |
| WD | tryptophan and aspartic acid |
| *wt* | wild-type |
| WT-1 | Wilms tumour-1 |
| YVAD-fmk | acetyl-Tyr-Val-Ala-Asp-fluoromethylketone |
| ZVAD-fmk | benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone |

## *Preface*

In accordance with the McGill Guidelines for Thesis Preparation, I am including the following excerpt to inform the external examiner of Faculty regulations:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidates's interest to make perfectly clear the responsibilities of all authors of the co-authored papers.

Included in this thesis are the texts from two published manuscripts and one manuscript in preparation.

**Chapter 2:** Chen, G., P.E. Branton, and G.C. Shore. 1995. Induction of p53-independent apoptosis by hygromycin B: suppression by Bcl-2 and adenovirus E1B 19-kDa protein. Exp Cell Res. 221:55-59.

In this paper, except the electron microscopy work was done by Kathy Teng, All the rest figures were done by myself. The establishment of stable cell lines expressing Bcl-2 or E1B 19kDa was assisted by Mai Nguyen.

**Chapter 3:** Chen, G., P.E. Branton, E. Yang, S.J. Korsmeyer, and G.C. Shore. 1996. Adenovirus E1B 19-kDa death suppressor protein interacts with Bax but not with Bad. J Biol Chem. 271:24221-24225.

All of the experiments in this paper were done by myself. The cDNA for Bad was kindly provided by Elizabeth Yang and Stanley Korsmeyer. Mai Nguyen, Joe Teodoro, and Richard Marcellus provided many helpful discussions

**Chapter 4:** Chen, G., P.E. Branton,, and G.C. Shore. 1999 (in preparation). Swapping the BH3 domain of Adenovirus E1B 19kDa protein with that of Bcl-2 converts 19K into a Bap31 and Bcl-xL associating protein

For this paper, the Flag tagged Bap31 expression construct was provided by Florence Ng. The immunofluorescence work of Figure 5 was assisted by Josée Lavoie. All the rest works for this chapter is my own.

## *Original Contributions to Knowledge*

1. Hygromycin B induces apoptosis.
2. The apoptosis induced by Hygromcyin B is p53-independent.
3. Adenovirus E1B 19kDa interacts with Bax.
4. The glycine at position 87 in the BH1 domain of E1B 19kDa is critical for its interaction with Bax.
5. Adenovirus E1B 19kDa does not interact with Bad.
6. E1B 19kDa contains an overlapping BH3/BH4 region.
7. E1B 19kDa does not interact with Bap31 nor Bcl-xL.
8. The BH3 domain of Bcl-2, when inserted into the E1B 19kDa protein (E1B 19kDa/Bcl2BH3), facilitates interactions with Bap31 or Bcl-xL.

# CHAPTER 1

## General Introduction

# *1.1 Overview of Programmed Cell death*

## 1.1.1 History

Apoptosis, or programmed cell death, is a genetically controlled and evolutionarily conserved cell death program that removes unwanted or potentially harmful cells. During apoptosis, individual cells are committed to self destruct without causing an inflammatory reaction which results in local tissue damage. Apoptosis is currently a major research focus in biochemistry, medicine, pharmacology, and many other fields because of its important role in development, tissue homeostasis, cancers, and many other diseases.

The concept of programmed cell death was first introduced by Glucksmann during his embryonic development studies in 1951 (Glucksmann, 1951). However, Flemming may be considered as the first person who observed and described the apoptosis phenomenon. Flemming noticed the degradation of nuclei and the condensation of chromatin in the epithelial lining cells of regressive ovarian follicles and named this phenomenon 'chromatolysis' (Flemming, 1885).

Apoptosis means "falling off" (such as leaves falling from trees, or petals from flowers) in Greek. It was formally introduced by Kerr, Wyllie, and Currie in 1972 to describe a unique form of cell death that they observed (Kerr et al., 1972). In the 'apoptotic death' process, cells do not show signs of cell swelling, cell membrane rupture, or release of internal contents into the environment, which are all features of necrotic cell death.

## 1.1.2 Apoptosis and Necrosis

Apoptosis is a highly regulated form of cell death that is morphologically and biochemically different from necrosis. The apoptotic process is associated with some unique cellular morphological changes, including plasma membrane blebbing, cell shrinkage, chromatin condensation and, in most cases, the cleavage of chromatin DNA into nucleosomal sized fragments (Kerr, 1971; Kerr et al., 1972). According to Kerr and Wyllie, the first visible sign of apoptosis is the condensation of chromatin into large masses

along the nuclear envelope along with membrane blebbing. Mitochondria and other organelles remain morphologically normal during the apoptotic process (Wyllie, 1980). Late events of apoptosis include the fragmentation of the cell into membrane-encapsulated " apoptotic bodies" containing intact organelles and other cell contents destined to be engulfed by macrophages and neighbouring cells (Kerr, 1971; Kerr et al., 1972).

Apoptotic cell death usually takes a longer period of time (Kroemer et al., 1998). Necrotic cell death, however, is a process that is often very rapid and violent, associated with cytoplasmic swelling, rupture of the cell membrane, and loss of integrity of subcellular and nuclear components (Kroemer et al., 1998; Wyllie, 1987; Wyllie, 1997). Physical or chemical damage can cause a number of cells in the tissue to die by necrosis. Necrosis usually results in localized inflammation because of the release of cell debris into the environment where the healthy neighbouring cells reside. Apoptosis, on the other hand, is a genetically determined process that functions in an individual cell. It is a natural, and essential process that happens during embryonic development, the establishment of the immune system, metamorphosis, and homeostasis. It causes little or no cell inflammation reactions (Henson and Johnston, 1987).

Apoptosis can be triggered by a variety of stimuli, such as viral infection (Teodoro and Branton, 1997; White and Gooding, 1994), growth factor withdrawal (Evan et al., 1992b; Sakamuro et al., 1995; Wagner et al., 1994), TNFα or Fas ligand (Nagata and and Golstein, 1995; Tewari and and Dixit, 1996), DNA damage (Clarke et al., 1993; Han et al., 1995; Long et al., 1997), and exposure to chemotherapeutic agents (Lowe et al., 1993). The time frame between when a cell receives a death signal and when commits itself to die varies from hours to days. However, the actual execution phase of cell death is rapid (about 30 min). The initial triggering phase of apoptosis may take different routes but the execution stage of apoptosis converges on a common pathway characterized by the activation of caspase cascades (Cryns and Yuan, 1998; Fraser and Evan, 1996; Oltvai and Korsmeyer, 1994; Takahashi and Earnshaw, 1996; White, 1996). Apoptosis can be divided into three different stages: (1) the induction stage; (2) the execution stage; and (3) the degradation stage. A variety of physiological and environmental stimuli, including cytokines, ionizing radiation, and chemotherapeutic drugs, among others, can induce apoptosis. During the induction

stage, cells receive death signals and commit themselves to die by apoptosis, but the cells are still morphologically and functionally normal. This stage has quite a variable length compared to the execution and degradation stages. The hallmark of the execution stage is the proteolytic process involving the activation of a family of cysteine proteases, the caspases (cysteinyl asparatate proteases), whose targets include the procaspases, the anti apoptotic protein Bcl-2/Bcl-xL, MDM2, Rb, Fodrin, Bap31, PKC δ and θ, MEKK-1, PARP (poly(ADP-ribose) polymerase),CAD(caspase-activated deoxyribonuclease)/DFF40/ CPAN, lamins, actins, and many other proteins (Enari et al., 1998; Halenbeck et al., 1998; Sakahira et al., 1998). The degradation stage is characterized by biochemical and morphological changes, including cell shrinkage, chromatin condensation and aggregation around the nuclear periphery, cell membrane blebbing, and formation of apoptotic bodies. One traditional method to distinguish apoptotic from necrotic cell death is by agarose gel electrophoresis of DNA from the dying cells. DNA from cells undergoing apoptosis is cleaved by endonuclease(s) to give nucleosomal size intervals (180-200bp) (Wyllie, 1980). These DNA fragments give a 'DNA ladder' pattern on the gel (Wyllie, 1980). At least one endonuclease, CAD (caspase-activated deoxyribonuclease) which is activated exclusively by caspases in response to apoptotic stimuli, has been isolated from murine lymphoma cells (Enari et al., 1998). A human homologue of CAD named CPAN has also been characterized in Jurkat cells (Halenbeck et al., 1998). A 45 kDa protein named DNA fragmentation factor (DFF45) has been isolated together with CAD and termed inhibitor of CAD (ICAD) (Liu et al., 1997; Sakahira et al., 1998). ICAD interacts with CAD in the cytosol and blocks CAD's endonuclease activity (Sakahira et al., 1998). During apoptosis, activated caspase-3 can effectively cleave ICAD into two fragments resulting in the release of which can then translocate to the nucleus and degrade chromosomal DNA (Enari et al., 1998; Sakahira et al., 1998).

Apoptotic cells do not always display all of the features of apoptosis, especially DNA fragmentation, which does not appear to be essential for cells to undergo apoptosis (Schulze et al., 1994). In fact, the fragmentation of DNA is only a consequence of apoptosis according to the recent ICAD/DFF45 murine knockout results (Zhang et al., 1998). The ICAD/DFF45 knockout mice are healthy and normal. Different types of cells from the

ICAD-/- mice can undergo the normal apoptotic process after appropriate stimuli, except that DNA fragmentation is completely abolished. This suggests that although ICAD/DFF45 is responsible for DNA fragmentation this event is not required for apoptosis (Zhang et al., 1998).

### 1.1.3 Apoptosis and Diseases

Likely all cells in multicellular organisms have the ability to undergo apoptosis. The inappropriate activation or inactivation of apoptosis has been linked to a variety of genetic and acquired diseases, including cancer, immune disorders, bacterial and viral infections, as well as neurodegenerative and neurodevelopemental disorders.

One of the breakthroughs that linked apoptosis to disease was the finding that B-cell follicular lymphomas have a t(14;18) chromosomal translocation (Cleary et al., 1986) which causes overexpression of the B-cell lymphoma-2 (Bcl-2) gene and , which has anti-apoptotic function. This translocation places Bcl-2 under the control of the constitutively expressed immunoglobulin heavy chain (Igh) promoter (Cleary et al., 1986). Although the mechanism of Bcl-2 function remains largely unknown, it is now the prototype of a large protein family with important regulatory functions in apoptosis. The identification of the role of Bcl-2 in B cell follicular lymphomas was very important because this was the first time that an oncogene had been functionally linked to the regulation of apoptosis rather than growth promotion. In CLL (chronic lymphocyte leukemia), lymphocytes express high levels of Bcl-2 causing the accumulation of excess non-dividing (G0 state) lymphocytes in the blood. Bcl-2 appears to keep these cells alive by preventing apoptosis. (Reed, 1998). Chemotherapeutic drugs and anti-neoplastic therapies, such as gamma radiation, mainly kill tumor cells by triggering the apoptotic cell death program rather than by killing the cells by direct structural damage, or necrosis (Fisher, 1994). The alteration of the apoptotic program in tumor cells is responsible for chemotherapeutic resistance in many cases. For example, Bcl-2 expression correlates with the poor response to chemotherapy in acute myeloid leukemia (Minn et al., 1995); and expression of Bcl-xL, a close family member of Bcl-2, correlates with resistance to chemotherapy in certain breast cancers (Campos et al.,

1993). In some colon tumors, frameshift mutations are found in the gene encoding Bax (Bcl-2 associated protein X), a pro-apoptotic member of Bcl-2 family which can antagonize Bcl-2's function (Rampino et al., 1997). By eliminating Bax, these tumor cells can survive as well as cells overexpressing Bcl-2 (Rampino et al., 1997). One example of an apoptosis related disease involves the human immunodeficiency virus (HIV), which causes an acquired immunodeficiency syndrome (AIDS) (Gougeon and Montagnier, 1993). HIV can induce apoptosis in both infected and uninfected CD4+ T cells which results in a massive depletion of T cells (Gougeon and Montagnier, 1993). Inappropriate apoptotic cell death has also been linked to neurodegenerative disorders, such as Alzheimer's, Huntington's, and Parkinson's disease; and to neurodevelopmental disorders, including Fragile X syndrome and schizophrenia (Margolis et al., 1994; Smale et al., 1995). Mutations in Fas and Fas ligand genes are associated with autoimmune-type disorders (Cohen and Eisenberg, 1991), and a heterozygous loss-of-function mutation in the Fas gene is linked to Hodgkin's lymphoma (Fisher et al., 1995).

## *1.2 The Cell Death Program Is Conserved Between C.elegans and Mammals*

Studies of *C.elegans* development in the past decade have made a very significant contribution to our understanding of programmed cell death in both *C.elegans* and mammals. Many genes involved in programmed cell death were first isolated from *C.elegans* because of the genetic simplicity and easy manipulation of the nematode (Hengartner, 1996; Hengartner et al., 1992; Hengartner and Horvitz, 1994). Horvitz and colleagues identified a number of genes involved in the cell death program in *C.elegans.* A homologous set of genes was also found in mammalian cells to be involved in the control of programmed cell death (Hengartner and Horvitz, 1994; Yuan et al., 1993; Zou et al., 1997).

At least three genes, ced-3, ced-4, and ced-9 (for *C.elegans* death gene 3, 4, and 9 respectively), are required for the control of the onset and execution of cell death in *C.elegans* (Hengartner et al., 1992; Yuan and Horvitz, 1992; Yuan et al., 1993). Of these

three genes, ced-3 and ced-4 are required for the promotion of cell death (Hengartner et al., 1992) while Ced-9 functions to inhibit cell death (Yuan et al., 1993).

Notably, all three genes, ced-3, ced-4, and ced-9, controlling *C.elegans* apoptosis, have mammalian homologues, suggesting that programmed cell death is a evolutionarily conserved pathway. Ced-9 shows sequence similarity to the mammalian anti-apoptotic protein Bcl-2 (Hengartner and Horvitz, 1994). Ced-3 is homologous to the mammalian interleukin-1-β-converting enzyme ( ICE ), the very first member of a large family of cysteine proteases now known as caspases (Yuan et al., 1993). Ced-4's first mammalian homologue termed Apaf-1 (apoptotic protease activating factor-1) was identified in 1997 (Zou et al., 1997). Two other mammalian Ced-4 homologues named CARD4 (Caspase recruitment domain 4) and FLASH ('FLICE-associated huge' protein) were identified in early 1999. (Bertin et al., 1999; Imai et al., 1999). The overall mechanism of apoptosis between *C.elegans* and mammals is very similar. However, the mammalian mechanism is more complicated and involves additional regulatory factors.

The Ced-4 protein acts between ced-9 and ced-3 in the *C.elegans* cell death pathway, implying that Ced-4 may function as an adapter protein between the two (Shaham and Horvitz, 1996). Ced-4 binds directly to both Ced-9 and Ced-3 and forms a ternary complex (Chinnaiyan et al., 1997). The current model is that Ced-3 is activated by the binding of Ced-4 to Ced-3 which stimulates Ced-3's autoproteolytic activity (Yang et al., 1998b). The Ced-4/Ced-3 interaction is essential for Ced-3 zymogen processing and mutations in Ced-4 that disrupt oligomerization prevent its ability to activate Ced-3 (Yang et al., 1998b). Ced-9 can inhibit cell death by directly binding to the Ced-3/Ced-4 complex possibly by preventing Ced-4 from forming oligomers; thereby preventing the autoproteolytic activation of Ced-3 (Yang et al., 1998b). Ced-4 binds to Ced-3 via a conserved N-terminal domain called a caspase-recruitment domain (CARD). ATP or dATP is required to facilitate Ced-3's auto proteolytic activity. A nucleotide-binding motif called a P-loop has been found in Ced-4. This P-loop is essential for the processing of Ced-3.

More recently, two *C.elegans* genes, ced-6 and ced-7, were found to be involved in the engulfment by surrounding cells during apoptosis (Liu and Hengartner, 1998; Wu and

Horvitz, 1998). Ced-7 is localized to the plasma membrane and shares sequence similarity to ABC (ATP binding cassette) transporters (Fath and Kolter, 1993; Wu and Horvitz, 1998). ABC transporters can mediate the transport of many substrates, including ions, phospholipids, and peptides across membranes (Fath and Kolter, 1993; Ruetz and Gros, 1994). The mammalian ABC transporter ABC1 is involved in the phagocytosis of apoptotic cells (Luciani and Chimini, 1996). Both sequence and functional similarities between Ced-7 and ABC1 transporter suggest they are homologues. Ced-6 contains a phosphotyrosine binding domain, a proline/serine rich region is also involved in the recognition and engulfment of apoptotic cells. Ced-6 might be an adapter molecule acting through a tyrosine kinase pathway (Liu and Hengartner, 1998).

A major contribution leading to the characterization of mammalian apoptotic regulatory proteins came from the isolation and characterization of three apoptosis protease-activating factors (Apaf-1, Apaf-2, and Apaf-3) from an apoptotic cell-free extract system (Li et al., 1997b; Liu et al., 1996; Zou et al., 1997). Apaf-1 is the first mammalian Ced-4 homologue. It is a 130 kDa protein sharing striking sequence similarity to Ced-4 (Zou et al., 1997). It contains a N-terminal CARD domain, a CED-4-like domain including the conserved nucleotide binding P-loop, which is responsible in Apaf-1 for binding of dATP, and WD (Tryptophan and Asparate) repeats, which are implicated in mediating protein-protein interactions, at the C-terminus (Zou et al., 1997). Apaf-2 was found to be cytochrome c, a 12kDa respiratory chain protein located in the mitochondrial intermembrane space and loosely associated with the inner mitochondria membrane. Cytochrome c is now known as a key regulator of the caspase cascade as it is an apoptotic trigger once released into the cytosol (Liu et al., 1996). Cytochrome c functions by binding to Apaf-1 and inducing a conformation change which promotes its interaction with caspase-9. Apaf-1 oligomerization is essential for the activation of caspase-9 (Srinivasula et al., 1998). Apaf-3 is caspase-9, an important initiator caspase whose activation can trigger an intracellular caspase cascade (Li et al., 1997b). Work done by Wang and colleagues also showed that the activation of caspase-3 from isolated cell-free extract requires cytochrome c, dATP, Apaf-1, and caspase-9 since depletion of any from the extract severely impairs caspase-3 activation (Li et al., 1997b; Liu et al., 1996). Based on their results and work from

other groups, Wang and colleagues proposed an apoptotic regulatory complex named the 'apoptosome', which consists of cytochrome c, dATP, Apaf-1, and caspase-9 (Li et al., 1997b). The binding of cytochrome c and dATP to the Apaf-1/caspase-9 complex can stimulate the proteolytic auto-activation of procaspase-9 via Apaf-1 oligomerization. Bcl-xL is thought to inhibit this autoproteolytic activation of procaspase-9 by binding to Apaf-1 and preventing oligomerization (Hu et al., 1998; Srinivasula et al., 1998). Although, Bcl-2 or Bcl-xL were not originally found in the 'apoptosome', a mammalian ternary protein complex like the *C.elegans* Ced-3, Ced-4, and Ced-9 complex has been proposed (Pan et al., 1998b) consisting of Apaf-1, Bcl-xL, and procaspase-9 (Pan et al., 1998b). Taken together, Ced-4 and Apaf-1 both function as adapter proteins to facilitate interactions between Ced-9/Ced-3 and Bcl-xL/procaspases respectively, to regulate the activation of the caspase cascade.

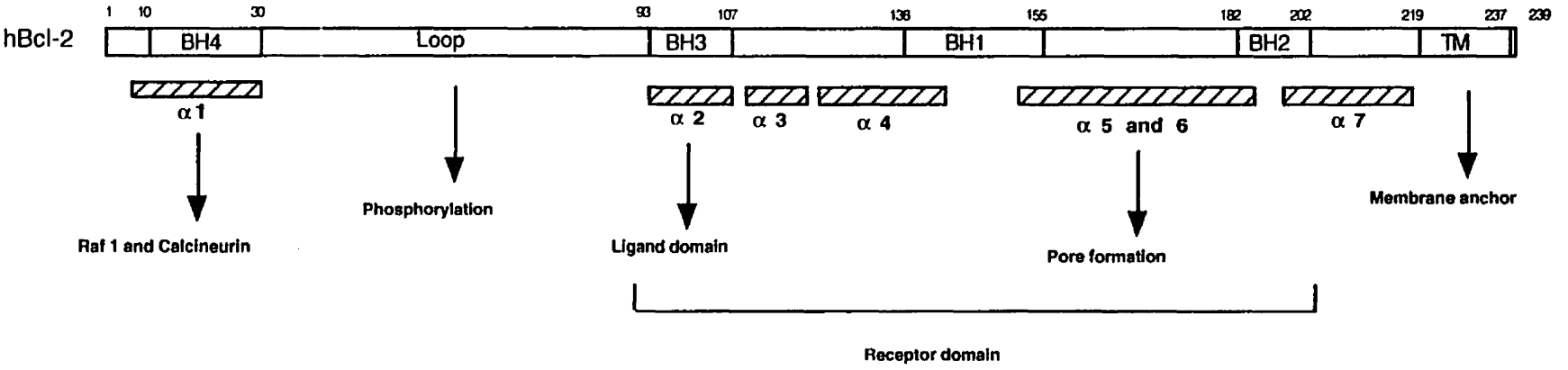
## *1.3 The Bcl-2 Protein Family*

### 1.3.1 Members of the Bcl-2 Protein Family

The anti apoptotic function of Bcl-2 was found by its ability to block the cell death of interleukin 3-dependent cells after the withdrawal of cytokines (Vaux et al., 1988). Upon IL-3 withdrawal, cells expressing high levels of Bcl-2 can enter a quiescent (G0) state of the cell cycle (Vaux et al., 1988). These cells can re-enter the cell cycle later upon addition of cytokines.

The human bcl-2 gene is very large because of a 225-kb intron splicing the coding sequence (Silverman et al., 1990), which consists of three exons (see Figure 1-1 on page 10). The level of bcl-2 mRNA is regulated at the transcriptional level and post-transcriptionally, since bcl-2 mRNA has a half-life of about 2-3 hours (Reed et al., 1987; Seto et al., 1988). The human bcl-2 transcript encodes a protein of 239 amino acids with a hydrophobic carboxy terminal region contributed by exon3. The mouse bcl-2 gene is highly homologous to human bcl-2 with a protein size of 236 amino acids (Negrini et al., 1987).

Figure 1-1 Bcl-2 structures.


hBcl-2
1
10
30
93
107
138
155
182
202
219
237
239
BH4
Loop
BH3
BH1
BH2
TM
α 1
α 2
α 3
α 4
α 5 and 6
α 7
Raf 1 and Calcineurin
Phosphorylation
Ligand domain
Pore formation
Membrane anchor
Receptor domain

Since the first cloning of the human gene bcl-2 at the breakpoint of the 14:18 translocation from human follicular lymphomas, a number of other bcl-2 related genes have been identified. The members of the Bcl-2 family of proteins have been extended to more than fifteen and the list is still growing (see Figure 1-2 on page 12). However, no equivalent genes from plants, Drosophila, or yeast have yet been found.

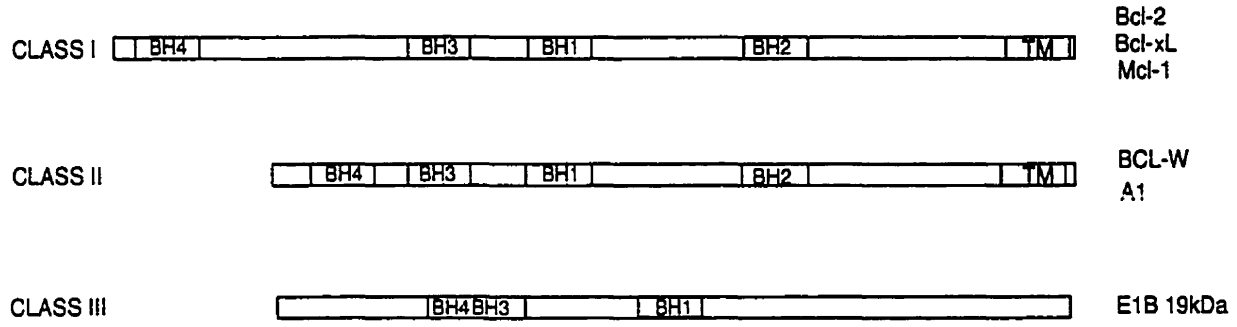
A bcl-2 related gene, bcl-x, was found shortly after the identification of bcl-2 (Boise et al., 1993). The Bcl-x gene encodes two protein products by alternative splicing of mRNA. They are Bcl-xL and Bcl-xS (Boise et al., 1993). They both carry the same c-terminal hydrophobic region as Bcl-2, but Bcl-xS has internal deletions of 63 amino acids. Like Bcl-2, Bcl-xL is an anti-apoptotic protein (Boise et al., 1993). Many cancer cells express high level of Bcl-xL. An intron splits the coding region of bcl-x at the same position as in bcl-2, indicating that they may have evolved by duplication (Boise et al., 1993).

Although Bcl-2 related proteins were first reported to function as anti-apoptotic proteins, subsequent studies show that some members of this family function as pro-apoptotic proteins. The truncated Bcl-xS protein functions in a dominant negative manner on both Bcl-2 and Bcl-xL. Bax, a Bcl-2 related protein, was found by its ability to interact with Bcl-2 in co-immunoprecipitation experiments (Oltvai et al., 1993). Bax is a pro-apoptotic protein (Oltvai et al., 1993) and its transient expression in cells is sufficient to induce apoptosis (Xiang et al., 1996). The bax gene is smaller than that of bcl-2. It consists of six exons. The exon 5/6 splice site within the coding region of bax corresponds precisely to the exon 2/3 splice within bcl-2 (Oltvai et al., 1993). Alternate splicing of bax exons produces three classes of transcripts whose levels vary in different tissues and cells. Only the 21-kDa bax protein can heterodimerize with Bcl-2 (Oltvai et al., 1993).

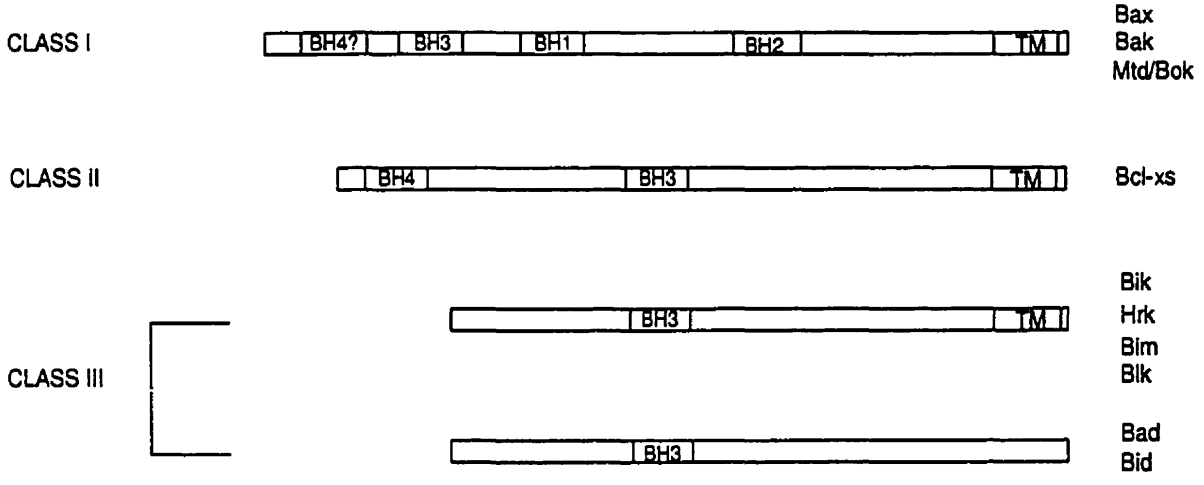
The Bcl-2 family can be divided into two subfamilies according to their function in apoptosis. Bcl-2 (Bakhshi et al., 1985; Cleary et al., 1986; Pegoraro et al., 1984; Tsujimoto et al., 1985; Tsujimoto and Croce, 1986), Bcl-xL (Boise et al., 1993), Bcl-w (Gibson et al., 1996), Bfl-1 (Choi et al., 1995), A1 (Lin et al., 1993), and Mcl-1 (Reynolds et al., 1994) act as death repressors (anti-apoptotic subfamily). The inducers of apoptosis (pro-apoptotic subfamily) includes Bad (Yang et al., 1995), Bax (Oltvai et al., 1993), Bik (Boyd et al., 1995),

Figure 1-2 Classification of Bcl-2 family of proteins.

## ANTI-APOPTOTIC SUBFAMILY




## PRO-APOPTOTIC SUBFAMILY



Bim (O'Connor et al., 1998), Bid (Wang et al., 1996c), Bak (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995), Blk (Hegde et al., 1998), Hrk (Inohara et al., 1997), and Bcl-xS (Boise et al., 1993). There are also many viral homologues of bcl-2, such as E1B 19kDa (Rao et al., 1992), BHRF1 (Henderson et al., 1993), ASFV 5HL (Neilan et al., 1993), HHV ORF16 (Nava et al., 1997), and HHV8bcl-2/KS-Bcl-2 (Russo et al., 1996), which function as apoptosis inhibitors during the viral infection of cells.

### 1.3.2 BH Domains and Bcl-2 Family of Proteins

A common feature of Bcl-2 family members is their ability to form either homo- or hetero-dimers with one another under certain conditions (Chao and Korsmeyer, 1998; Oltvai et al., 1993; Yang and Korsmeyer, 1996). Two motifs named BH1 and BH2 (Bcl-2 homology region 1 and 2), which are 18 and 16 amino acid residues in length respectively, are highly conserved and exist in most Bcl-2 anti-apoptotic family members. BH1 and BH2 are required for homodimerization and heterodimerization of Bcl-2 family proteins (Chao and Korsmeyer, 1998; Oltvai et al., 1993; Yang and Korsmeyer, 1996). The BH1 and BH2 regions are also required for the anti-apoptotic functions of Bcl-2 (Oltvai et al., 1993). Mutation of the conserved glycine residue within the BH1 domain, which is one of the residues forming the hydrophobic groove in Bcl-2, can both inhibit Bcl-2's anti-apoptotic function and block its heterodimerization with Bax (Yin et al., 1994). Some Bcl-2 family members have ion conducting channel forming ability (Minn et al., 1997). This ability of Bcl-2/Bcl-xL or Bax to form such channels in synthetic lipid bilayers also requires the BH1 and BH2 regions (Schendel et al., 1997). A third 16 amino acid residue conserved region called the BH3 domain was first defined in Bak for its essential role in heterodimerization between Bak and Bcl-2, and its role in death promotion (Chittenden et al., 1995). The importance of the BH3 domain is discussed in the next section. The fourth conserved domain, called BH4, was defined recently. It is a 21 amino acid residue region located at the N-terminus of some anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Ced-9 (Huang et al., 1998). This domain is implicated in protein-protein interactions. Several proteins including Ced-4 (Huang et al., 1998), Apaf-1 (Pan et al., 1998b), calcineurin (Shibasaki et al., 1997), and Raf-1 (Wang et al., 1994a) interact with Bcl-2

through the BH4 domain. A BH4 deletion mutant of Bcl-2 loses its anti-apoptotic function (Huang et al., 1998).

### 1.3.3 BH3, The Killer Domain

Bcl-2 and Bcl-xL contain all four BH domains (BH1, BH2, BH3, and BH4), suggesting that the anti-apoptotic members require the presence of more than one BH domain to exert their anti-apoptotic function. Some death agonist members of the Bcl-2 family of proteins, like Bad, Bik, Bim, Bid, Blk, and Hrk contain only a BH3 domain suggesting that the BH3 domain is necessary and sufficient for their pro-apoptotic activity.

The BH3-only pro-apoptotic members share no sequence homology with other Bcl-2 family members except the BH3 region. The BH3-only pro-apoptotic Bcl-2 family of proteins can form heterodimers with anti-apoptotic family members but can not form homodimers indicating that the BH3 domain alone is sufficient for heterodimerization. Bid is the only BH3-only pro-apoptotic member which can bind to both pro-apoptotic members Bax and Bak as well as to the anti-apoptotic members (Wang et al., 1996c). The importance of this dual binding ability is not clear. It seems that the BH3 domain from the pro-apoptotic Bcl-2 family members has two functions. One is that it mediates the interaction with anti-apoptotic members. The other is that it is also required for the apoptotic induction function of these BH3-only pro-apoptotic members and in this regard has been termed the 'Killing Domain'.

Pro-apoptotic members Bax, Bak, and Mtd/Bok contain BH1, BH2, BH3, and possibly BH4 domains. These pro-apoptotic members can form homodimers and heterodimers with anti-apoptotic members suggesting that homodimerization requires additional BH domains other than BH3. However, it is still the BH3 domain that is important for their heterodimerization with anti-apoptotic members and their pro-apoptotic activity. A detailed comparison of BH3 domains from both pro-and anti- apoptotic members of the Bcl-2 family shows that the BH3 domain in anti-apoptotic members can act as a death promoter similar to BH3 domains in pro-apoptotic members. This raises the question of how Bcl-2 and Bcl-xL can contain a death promoting BH3 domain and still be anti-

apoptotic. One possibility is that the BH4 domain (and probably the loop region located between BH3 and BH4) of Bcl-2 and Bcl-xL can inhibit the BH3 domain's pro-apoptotic function.

### 1.3.4 Gene Deletion Studies

Despite the structural and functional similarities between Bcl-2 and Bcl-xL, there is evidence that the two proteins may function differently in some systems. For example, ectopic expression of Bcl-xL can block apoptosis in a murine B-cell line, WEHI-23, but Bcl-2 fails to do so (Gottschalk et al., 1994). The Bcl-2 and Bcl-xL knockout mice have also indicated functional differences between the two proteins. The Bcl-2-/- mice are viable and normal with the exception of a lack of retinal development, indicating that Bcl-2 is not essential for mouse embryonic development (Veis et al., 1993). In contrast to Bcl-2, the Bcl-xL knockout results in embryonic lethality (Motoyama et al., 1995). The Bcl-xL-/- mouse embryos die at around day 13 due to excessive cell death in the brain and other systems (Motoyama et al., 1995). Bcl-xL appears to be essential for brain development (Motoyama et al., 1995). Bax -/- mice represent the first knockout of a death-promoting family member (Knudson et al., 1995). The Bax knockout mice look normal but develop both hyperplasia and hypoplasia depending on the cellular context (Knudson et al., 1995). Bax-deficient primary fibroblasts showed resistance to chemotherapy and p53-induced apoptosis, indicating that Bax may function as a tumor suppressor gene (Knudson et al., 1995).

### 1.3.5 The Structure of Bcl-xL and Other Bcl-2 Family Proteins

The crystal and solution structures of Bcl-xL have been solved (Muchmore et al., 1996). Based on X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR), the Bcl-xL structure appears largely α-helical, with the BH1, BH2, and BH3 domains together forming a hydrophobic cleft (Muchmore et al., 1996). The NMR structure of Bak indicates that the BH3 domain interacts with the hydrophobic cleft formed by the BH1, BH2, and BH3 domains of Bcl-xL. Interestingly, Bik, Bad, Bid, and Hrk, which contain only a BH3

domain, are more potent death effectors than those proteins with all of the BH domains (Bax, Bak, and Bok) (Muchmore et al., 1996). It is not clear whether binding of the BH3 domain of the death agonist inactivates Bcl-2/Bcl-xL by simple stoichiometric binding or if it initiates a new activity that triggers apoptosis. A 60 amino acid loop region located in the N-terminal region between the first and second α-helices (the BH3 and BH4 domains) of Bcl-xL is structurally undefined (by X-ray) suggesting that this region is very flexible (Muchmore et al., 1996). Deletion studies show that this region is nonessential for the anti-apoptotic effect of Bcl-xL. Instead, this region may serve as a site for regulation of Bcl-2/Bcl-xL. Ser/Thr phosphorylation of some residues within this loop region have been found to modulate the function of Bcl-2 (Haldar et al., 1998).

The structure of Bcl-xL is similar to bacterial pore-forming proteins; the colicins (A1 and E1) and diphtheria toxin, which have pH-dependent membrane-pore forming ability (Muchmore et al., 1996). Furthermore, Bcl-xL/Bcl-2 and Bax form ion channels in synthetic phospholipid bilayers in a pH-dependent manner just as bacterial toxins do (Minn et al., 1997; Schendel et al., 1997). Two hydrophobic helices, α5 and α6, flanked by the BH1 and BH2 domains, are responsible for the channel formation ability of Bcl-2/Bcl-xL and Bax (Schendel et al., 1997). Thus Bax, Bcl-xL and its close relative Bcl-2, may directly or indirectly affect the permeability and/or transport capacity of organelles and regulate their homeostasis during apoptotic processes. Indeed, Bcl-2 inhibits the release of holocytochrome c and an apoptosis inducing factor (AIF) from the intermembrane space of mitochondria into the cytoplasm (Susin et al., 1997; Susin et al., 1996; Yang et al., 1997). Ion channel formation experiments in vitro using synthetic phospholipid bilayers show that anti-apoptotic family members like Bcl-2 and Bcl-xL have different ion selectivity and conduction ability than the pro-apoptotic family member Bax (Schlesinger et al., 1997). The Bcl-2 and Bcl-xL channel is pH sensitive and becomes cation selective at physiological pH (Minn et al., 1997). The Bax channel prefers anions such as Cl-, and functions over a broad pH range (Schlesinger et al., 1997). Bcl-2 also inhibits Bax channel-forming activity rather than forming its own ion channel. However, all of these results come from in vitro systems and no data demonstrates that Bcl-2/Bcl-xL or Bax have ion channel formation properties in the cell under physiological conditions.

## *1.4 The Functional Mechanism(s) of the Bcl-2 Family of Proteins*

Despite rapid progress in the apoptosis field, the exact mechanism(s) by which Bcl-2 family members regulate apoptosis remains largely unclear.

Overexpression of Bcl-2 and other anti-apoptotic members in many mammalian cells can block apoptosis induced by a broad range of stimuli (Gajewski and Thompson, 1996; Reed, 1996). Bax and the other pro-apoptotic members of the Bcl-2 protein family function by accelerating apoptosis. Bax can heterodimerize with the anti-apoptotic members like Bcl-2 (Oltvai et al., 1993). The ratio of Bcl-2 to Bax in the cell is important for the apoptotic outcome (Oltvai et al., 1993). When Bcl-2/Bcl-xL are dominant, the cell survives death triggers. Conversely, when Bax, or other pro-apoptotic members, are expressed at high level, the cell undergoes apoptosis.

Many studies show that Bcl-2 can act through several mechanisms. It can act as an antioxidant (Hockenbery et al., 1993), can prevent mitochondrial release of cytochrome c (Kharbanda et al., 1997; Yang et al., 1997), and can delay cell proliferation by prolonging the G1 phase of the cell cycle (Reed, 1996). Perhaps most importantly, Bcl-2 can prevent the activation of a subclass of cysteine proteases called caspases (formerly known as ced-3/ICE (interleukin-1-converting enzyme)-like proteases) (Boulakia et al., 1996; Yang et al., 1997). The anti-apoptotic activity of Bcl-2 seems to be proportional to its expression level (Oltvai et al., 1993). Subcellular localization studies of Bcl-2 reveal that Bcl-2 is anchored via its carboxyl-terminal hydrophobic region to the outer membranes of mitochondria, nuclei, and the endoplasmic reticulum leaving the bulk of the protein facing the cytoplasm (Chao and Korsmeyer, 1998; Hockenbery et al., 1990; Nguyen et al., 1993). A cytosolic Bcl-2 mutant only partially retains its anti-apoptotic protection function suggesting that membrane localization is required for full activity (Nguyen et al., 1994). Bax is reported to be an integral membrane protein associated with organelles, or bound to organelles in cells undergoing apoptosis, but in other work, Bax has been found to be a soluble protein in the cytoplasm (Goping et al., 1998; Gross et al., 1998). The Bax monomer exists in the cytosol and the homodimer form resides in mitochondria and other membrane locations upon

death triggering (Goping et al., 1998; Gross et al., 1998). Bax can homodimerize only when it is targeted to mitochondrial outer membrane (Goping et al., 1998; Gross et al., 1998; Wang et al., 1998). A mechanism for Bax targeting to mitochondria was proposed recently. The N-terminal region of Bax contains an ART (for apoptosis regulation of Bax targeting) domain, which is a mitochondria targeting inhibition domain. Upon apoptotic stimulation, an unidentified cytosolic protein named Baf (Bax associated factor) can interact with Bax and suppress the mitochondria targeting inhibition effect of ART (Goping et al., 1998). Bax then can translocate onto the mitochondria outermembrane to interact with other mitochondria outer membrane proteins, such as Bcl-2/Bcl-xL and probably truncated Bid to exert its pro-apoptotic functions (Li et al., 1998; Luo et al., 1998). Bid, a BH3-only pro-apoptotic member, which can interact with both Bcl-2 and Bax, is also targeted to mitochondria after being proteolytically cleaved by caspase-8 in cells that are undergoing apoptosis (Li et al., 1998; Luo et al., 1998). Forced dimerization of Bax causes Bax to translocate from the cytosol to mitochondria membranes (Gross et al., 1998). This translocation can result in mitochondrial dysfunction and induce apoptosis (Gross et al., 1998).

To understand the mechanism(s) by which Bcl-2 functions, it is necessary to identify the molecules with which Bcl-2 interacts. A variety of techniques, including interaction cloning, yeast two-hybrid and recombinant baculovirus systems, have attempted to isolate Bcl-2 interacting proteins. Some binding partners of Bcl-2 and Bax have been identified. Among them are c-Raf-1, a protein kinase that transduces growth factor-elicited signals via the mitogen-activated protein kinase pathway, (Wang et al., 1996a; Wang et al., 1996b) (34-36); BAG-1 (Takayama et al., 1995), and calcineurin (Shibasaki et al., 1997). These proteins have been isolated based on their ability to interact with Bcl-2 or Bcl-xL, but their ability to function as apoptosis regulators under in vivo physiological conditions needs to be established. Three proteins interacting with the adenovirus protein E1B 19kDa were isolated: Nip-1, 2, and 3 (nineteen kDa interacting protein-1, -2, and –3 (Boyd et al., 1994). They are also able to interact with Bcl-2 (Boyd et al., 1994). Nip-3 contains a BH3 domain and was renamed BNIP3 as a pro-apoptotic member of the Bcl-2 family of proteins

(Yasuda et al., 1998). The targeting of Nip-3 to mitochondria is essential for its ability to induce apoptosis in the cell (Chen et al., 1997).

### 1.4.1 Homo- and Hetero-dimerization

Homo-and hetero-dimerization are common features of all members of the Bcl-2 family of proteins. Many studies suggest that the dimerization process is essential for the biological activity of these proteins. Mutagenesis studies show that the anti-apoptotic activity of Bcl-2 correlates with its ability to form heterodimers with Bax through BH1 and BH2 domains (Yin et al., 1994). BH1, BH2, and BH3 domains of Bcl-2 can also interact with the BH3 domain of Bax and prevent Bax from inducing apoptosis (Yin et al., 1994). In this case, the survival/death activity and heterodimerization capacity are non-separable functions. A Bcl-xL mutant, which has lost heterodimerization ability with Bax or Bak, retains its anti-apoptotic function, suggesting that the dimerization of Bcl-xL with Bax is not required (Cheng et al., 1996). Studies of Bcl-2 and Bax knockout mouse, also demonstrate that Bax can promote apoptosis in the absence of Bcl-2 and Bcl-2 can prevent apoptosis independent of Bax, suggesting that Bcl-2/Bax heterodimerization may not be the only mechanism that Bcl-2 uses to regulate apoptosis (Knudson et al., 1995). Some evidence shows that Bax mutants that have lost both hetero- and homodimerization ability can still trigger apoptosis suggesting that both homo- and hetero-dimerization properties are not required for its death promoting ability as determined by yeast two-hybrid and detergent-based IP assays (Simonian et al., 1996). However, detailed studies of the same Bax mutants show that they are located predominantly in the mitochondrial membrane and can be cross-linked to form homodimers just as readily as wild type Bax (Wang et al., 1998). This observation suggests that Bax undergoes a conformational change when translocated from the cytosol to the mitochondrial membrane and this conformational change is essential for its homo- and hetero-dimerization properties, and its death promoting function (Wang et al., 1998).

Together, these findings suggest two models for the regulation of apoptosis by Bcl-2 and Bax. First, Bcl-2 and Bax function independently to interact with survival and death

promoting molecules respectively, but can neutralize each other's function through heterodimerization. Secondly, the Bax homodimer is the active form triggering apoptosis in the cell. Bcl-2 can form heterodimers with Bax to protect cells from apoptosis. In either case, the level of Bax and Bcl-2 heterodimers can act as a setpoint in the cell to determine whether the cell should survive or die. The intracellular protein levels of Bcl-2 and Bax can adjust this setpoint.

## 1.4.2 Regulation of Caspase Activation

### 1.4.2.1 Bcl-2 and Caspase Activation

Two main mechanisms have been proposed to link Bcl-2 to caspase activation. First, Bcl-xL and caspase-9 can bind distinct regions of Apaf-1 to form a ternary complex in mammalian cells (Pan et al., 1998b). The binding of Bcl-2 to Apaf-1 can prevent Apaf-1 from causing proteolytic activation of procaspase-9 and thereby inhibit the activation of the caspase cascade (Srinivasula et al., 1998). Secondly, Bcl-2 can function by inhibiting the release of caspase activators such as cytochrome c and AIF (apoptosis-inducing factor) from mitochondria (Kim et al., 1997; Kluck et al., 1997). The release of cytochrome c from mitochondria is a key event that leads to the amplification of caspase activation (Liu et al., 1996).

### 1.4.2.2 Bcl-2 and Caspase-9 Autocatalytic Activation

In the first model, through the Apaf-1 adapter molecule, Bcl-2/Bcl-xL, can recruit procaspase-9 and other initiator procaspases to intracellular membrane locations, such as mitochondrial outer membrane and the ER. This prevents caspases from being activated. Apaf-1 can form oligomers (Srinivasula et al., 1998), and can induce the activation of procaspase-9 associated with Apaf-1 by cleavage at Asp-315 by an intrinsic autocatalytic activity of procaspase-9 (Srinivasula et al., 1998). Deletion of WD-repeats, a protein-protein interaction motif within Apaf-1, can render Apaf-1 constitutively active and cause the autoproteolytic processing of procaspase-9, independently of cytochrome c and dATP.

This result indicates that the WD repeats are involved in the regulation of Apaf-1 activity via cytochrome c and dATP (Srinivasula et al., 1998). This cytochrome c regulation step is unique for mammals since Ced-4 in *C.elegans* does not contain the WD repeat motif and does not require cytochrome c.

Apaf-1 knockout mice die at embryonic day 16.5. Virtually all tissues and cells whose development involves programmed cell death have been disturbed, including the formation of the palate, neural cells, the removal of interdigital webs, and lens and renal development (Cecconi et al., 1998; Yoshida et al., 1998). Fibroblasts from Apaf-1-/- mice exhibit a reduced response to various apoptotic stimuli (Cecconi et al., 1998; Yoshida et al., 1998). This clearly demonstrates the essential role of Apaf-1 in tissue and organ development. The Apaf-1 knock out clearly demonstrates that developmental cell death is dependent on cytochrome c release from mitochondria and emphasizes the importance of the apoptosome in mammalian development.

#### 1.4.2.3 Bcl-2 Family and Release of Apoptotic Inducers

In the second model, evidence suggests that Bcl-2, Bcl-xL, and Bax may be pore-forming proteins similar to bacterial toxins such as diphtheria toxin (London, 1992; Muchmore et al., 1996; Parker and Pattus, 1993). The ion conducting channels formed by Bcl-xL or Bax have opposite ion selection properties; and Bcl-xL can inhibit Bax ion channel forming ability (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). All these data suggest that Bcl-2, Bcl-xL, and Bax may also have a membrane transport function that regulates ion flux and protein transport across the mitochondrial membrane, the endoplasmic reticulum, and nuclear envelope. In support of this model, Bcl-2 and Bcl-xL can inhibit the release of cytochrome c and AIF (apoptosis-inducing factor) from mitochondria, whereas, Bax promotes cytochrome c release.

#### 1.4.2.4 Regulation of Ced-3 Activity by Ced-9

The function of the trimeric protein complex Ced-3/Ced-4/Ced-9 in *C.elegans* is also emerging. Ced-3 can undergo an autoproteolytic cleavage by a mechanism similar to that

of mammalian Apaf-1 induced procaspases via association with the Ced-4 oligomers (Yang et al., 1998b). The oligomerization domain within Ced-4 has been mapped to amino acids 171-549. The oligomerization of Ced-4 can bring associated Ced-3 molecules into proximity with each other thereby inducing autoproteolytic processing of Ced-3 (Yang et al., 1998b). Other regions of Ced-4, including the N-terminal CARD domain that interacts with the CARD of Ced-3, and the D-loop region that is responsible for Ced-4 nucleotide binding, are non-essential for the Ced-4 oligomerization process (Yang et al., 1998b). Co-expression of truncated Ced-4 (172-549), Ced-4, Ced-3, and Bcl-xL, can prevent Ced-4-Bcl-xL interaction but has no effect on Ced-4 dimerization, suggesting that Ced-4 oligomerization and Ced-4 binding to Bcl-xL are mutually exclusive events. Similarly, the same loss of function Ced-4 mutant can still bind Ced-3 but does not allow Ced-3 activation since the molecules are not brought together (Yang et al., 1998b). Thus, Ced-3 activation in the Ced-3/Ced-4 complex is controlled by Ced-4 oligomerization, and is regulated by interaction with Ced-9 or Bcl-xL (Yang et al., 1998b).

#### 1.4.2.5 Inhibition of Caspase Activation by Bcl-2 and Ced-9

In both *C.elegans* and mammals, BH3-only pro-apoptosis proteins can disrupt the proteolytic autoprocessing of Ced-3/procaspase. EGL-1 is a BH3-only *C.elegans* protein, and can effectively replace Ced-9 from the Ced-3/Ced-4 complex and thus promote auto activation of Ced-3 (Conradt and Horvitz, 1998). Similarly, Bid, a mammalian BH3-only pro-apoptotic member, can disrupt Bcl-xL association with Apaf-1/procaspase-9 and also cause procaspase-9 activation (Luo et al., 1998). The BH3 domains of EGL-1 and Bid interact with the hydrophobic cleft formed by BH1, BH2, and BH3 on Ced-9 or Bcl-xL and somehow cause the release of Ced-9/Bcl-xL from the Ced-3/Ced-4 or Apaf-1/procaspase-9 complexes. In summary, Bcl-2/Ced-9 promotes cell survival by inhibiting the binding of Ced-4 adapter proteins needed for caspase activation. Those pro-apoptotic Bcl-2 family members, like EGL-1 and Bid, apparently promote cell death through mechanisms that include disruption of Bcl-2/Ced-9 association with the adapters.

### 1.4.3 Regulation of Bcl-2/Bcl-xL Activity by Caspases

The anti-apoptotic proteins Bcl-2 and Bcl-xL contain caspase cleavage sites in their loop region located between the BH3 and BH4 domains. Both Bcl-2 and Bcl-xL can be proteolytically cleaved by caspases at these sites (Clem et al., 1998; Grandgirard et al., 1998). Once cleaved, Bcl-2 and Bcl-xL lose their anti-apoptotic function (Clem et al., 1998; Grandgirard et al., 1998). Truncated Bcl-2 and Bcl-xL proteins can promote cell death (Clem et al., 1998; Grandgirard et al., 1998). These results suggest that when death suppression is no longer an option for the cell, Bcl-2 and Bcl-xL can function as a death inducer after being cleaved by the caspases.

### 1.4.4 ER Regulation of Apoptosis

Although the localization of Bcl-2 to the ER and nucleus was observed a long time ago, the role Bcl-2 plays at these locations has not been addressed extensively. Bap31, an integral ER protein, was isolated based on its ability to interact with Bcl-2 in farwestern analysis (Ng et al., 1997). In transiently transfected 293T cells, Bap31 is a part of an ER membrane protein complex that includes Bcl-xL/Bcl-2, caspase-8, and a Ced-4 homologue (Ng et al., 1997; Ng and Shore, 1998). The efficient interaction of Bap31 with Bcl-xL requires the presence of an endogenous Ced-4 homologue suggesting that an ER caspase activating complex similar to that of the mitochondrial "apoptosome" and cell membrane DISC may exist (Ng and Shore, 1998). Bax is not present in this ER-Bap31 complex, in fact, Bax can effectively replace Bcl-2 or Bcl-xL from the complex and induce apoptosis (Ng and Shore, 1998).

### 1.4.5 Bad and Its Function in Apoptosis

Bad is a pro-apoptotic member of the Bcl-2 family of proteins. Bad is able to induce apoptosis when transfected into mammalian cells (Yang et al., 1995). Bad was first isolated from a mouse cDNA library based on its interaction with the anti-apoptotic protein Bcl-2 (Yang et al., 1995). It was subsequently shown to interact more strongly with Bcl-xL than

with Bcl-2, and in functional studies it antagonizes the protective effect of Bcl-xL effectively. The human homologue of Bad has also been cloned (Ottilie et al., 1997). Bad does not homodimerize with itself. It functions by binding to anti-apoptotic proteins such as Bcl-xL and Bcl-2. In fact, Bad so strongly interacts with Bcl-2, that it can effectively liberate Bax from Bcl-2/Bax heterodimers and produce the free form of Bax which can induce apoptosis (Yang et al., 1995). Bad is localized in the cytosol and on subcellular membranes (Zha et al., 1996b). Furthermore, translocation of Bad between cytosol and membranes has been observed (Zha et al., 1996b). The translocation is regulated by phosphorylation of Bad (Zha et al., 1996b). Bad is rapidly phosphorylated on both Ser112 and Ser136 in response to growth promoting factors (Zha et al., 1996b). These two serine sites match the consensus binding sites for the cytosolic phosphoserine binding protein 14-3-3. Phosphorylated Bad can bind to the 14-3-3 chaperone protein and be sequestered in the cytosol in a complex with 14-3-3 (Zha et al., 1996b). When cells receive death stimuli, Bad is rapidly dephosphorylated and dissociated from 14-3-3 (Zha et al., 1996b). The unphosphorylated Bad can move to the mitochondrial membrane to interact with Bcl-2/Bcl-xL and other membrane-anchored family members (Zha et al., 1996b). The Bad and Bcl-2/Bcl-xL interaction can readjust the death setpoint formed by Bcl-2/Bcl-xL and Bax by shifting it towards apoptosis (Yang et al., 1995; Zha et al., 1996b). Mutation of Ser112 and Ser136 phosphorylation sites in Bad can abrogate its binding to 14-3-3 and enhance its pro-apoptotic activity (Zha et al., 1996b).

Several growth factors including IL-3 can induce the activation of the phosphatidylinositol 3-kinase (PI3K) (Del et al., 1997). The activated PI3K mediates a variety of cellular responses, including cell growth and apoptosis, by generating PtdIns(3,4)P2 and PtdIns(3,4,5)P3. These 3-phosphoinositides then function directly as second messengers to activate downstream signaling molecules by binding pleckstrin homology (PH) domains in these signaling molecules (Rameh et al., 1997). AKT (also known as protein kinase B, PKB) is a good example of a PH domain-containing protein that is regulated by binding PI3K products (Franke et al., 1997). Binding of the PH domains of AKT to PtdIns(3,4)P2 or PtdIns(3,4,5)P3 functions to relocalize AKT from the cytosol to the plasma membrane (Downward, 1998). Targeting AKT to the plasma

membrane allows AKT to be phosphorylated and activated by the protein kinase 3-phosphoinositide-dependent protein kinase(PDK)1 (Downward, 1998). AKT is a serine/threonine kinase. Once being activated by PI3K, AKT can phosphorylate Bad at Ser136. The phosphorylated Bad associates with 14-3-3 and is sequestered in the cytosol. (Datta et al., 1997; del et al., 1997). The phosphatase which responds to death signals by dephosphorylating Bad is still unknown. PTEN (also known as MMAC1 and TEP1), a tumor suppressor with sequence homology to protein tyrosine phosphatases and the cytoskeletal protein tensin (Li and Sun, 1997; Li et al., 1997a; Steck et al., 1997), can negatively regulate Akt's activity (Stambolic et al., 1998). A constitutively active mutant of Akt can rescue cells from PTEN-induced apoptosis, indicating that PTEN is a negative regulator upstream of Akt (Stambolic et al., 1998).

The BH3 domain of Bad is necessary for its interaction with Bcl-2 and Bcl-xL (Zha et al., 1997). Early studies suggested that there are weak BH1 and BH2 homologous domains existing in Bad. More recent arguments show that the BH3 domains of BCL-2 family proteins are homologous to previously hypothesised BH1 and BH2 domains of Bad (Zha et al., 1997). The current understanding is that there are no BH1 and BH2 domains in Bad. Bad contains an amphipathic BH3 domain only and lacks the hydrophobic C-terminal region possessed by other membrane-based members of the Bcl-2 family of proteins. This may explain why Bad does not form homodimers since BH1 and BH2 domains are required for all homodimerization processes and Bad contains neither BH1 or BH2. Peptides derived from the Bad BH3 domain can completely inhibit the dimerization of Bad with Bcl-2 or Bcl-xL, indicating that the BH3 domain of Bad is necessary for its interaction with anti-apoptotic proteins such as Bcl-2 or Bcl-xL (Ottilie et al., 1997; Zha et al., 1997). However, the interaction between Bcl-2 and Bad may require structural information other than the BH1, BH2, and BH3 domains. Several other anti-apoptotic Bcl-2 family members, including Mcl-1, A1, and E1B 19kDa, fail to interact with Bad suggesting that Bad regulates some of the anti-apoptotic Bcl-2 family members, but not all (Yang et al., 1995). A E1B 19kDa chimeric protein bearing the BH1, BH2, and BH3 domains from Bcl-2 does not interact with Bad, indicating that structural features other than the BH domains of Bcl-

2/Bcl-xL are required for the interaction (Gang Chen and Gordon Shore, unpublished data).

### 1.4.6 Mitochondria Targeting of Bax and Regulation of Apoptosis

Bax can translocate from cytosol into the mitochondrial membrane in response to death stimuli (Wolter et al., 1997). Once translocated onto the mitochondrial membrane, Bax can form homodimers. This homodimerization process is important for Bax's pro-apoptotic function and its ability to induce cytochrome c release from mitochondria, suggesting that a specialized intramembrane conformation of Bax is required for its pro-apoptotic activity (Gross et al., 1998; Wang et al., 1998). The translocation of Bax from cytosol to mitochondria requires the help of a yet-to-be-identified cytosolic factor as well as mitochondrial outer membrane docking protein(s). The N-terminal of Bax contains an inhibitary domain, ART (for apoptotic regulation of targeting) that prevents Bax from auto translocating into the mitochondrial membrane (Goping et al., 1998). In vitro targeting experiments show that deletion of ART can target Bax to mitochondria without the requirement of apoptotic cell extracts (Goping et al., 1998). The putative cytosol factor Baf (for Bax activating factor) can interact with Bax and target Bax to mitochondria (Goping et al., 1998).

### 1.4.7 Cytochrome c Release Induced by Bid

In addition to Bad, two more pro-apoptotic Bcl-2 family members, Bax and Bid, can translocate from cytosol into mitochondrial membranes after cells receive death signals. Bid was recently purified from Hela cell S-100 cytosolic fraction and identified as the factor that can efficiently induce the release of cytochrome c from mouse liver mitochondria (Luo et al., 1998). The ability of Bid to induce cytochrome c release requires the presence of caspase-8 activity (Li et al., 1998; Luo et al., 1998). Bid is a preferred caspase-8 substrate (Li et al., 1998). The truncated form of Bid (cleaved by caspase-8 at the Bid LQTD caspase recognition site) but not full length Bid, was translocated to the mitochondrial membranes. Truncated Bid is the active form of Bid that induces cytochrome c release from

mitochondria. Bid contains none of the BH1, BH2, BH3, BH4 domains, or the c-terminal hydrophobic region. The only sequence homology Bid shares with other pro-apoptotic Bcl-2 family members is the BH3 domain. The 15-kDa caspase-8 cleavage product of Bid, aa 60-195, contains the BH3 domain, a domain that can mediate direct interaction with anti-apoptotic members such as Bcl-2 and Bcl-xL. A Bid G94E BH3 mutant that lost its ability to interact with Bcl-2/Bcl-xL is defective in cytochrome c release but can still translocate into mitochondrial membrane (Luo et al., 1998). This means that Bid interacts with an unidentified mitochondrial membrane protein since this protein-protein interaction itself is not enough to trigger cytochrome c release. The ability of truncated Bid to induce cytochrome c release may require its association with this unidentified protein to interact with Bcl-2/Bcl-xL or other members of the Bcl-2 family which are located on the mitochondrial outermembrane and inhibit release of cytochrome c.

### 1.4.8 Comparison of the Mechanisms Used by Bad, Bax, and Bid

All three pro-apoptotic proteins, Bad, Bax, and Bid, use cytosol to membrane translocation strategies to exert their pro-apoptotic functions. but each of them uses a different mechanism for the translocation process. For Bad, it is the phosphorylation that mediates the translocation (Datta et al., 1997; del et al., 1997; Zha et al., 1996b); for Bid, it is the proteolytic cleavage of Bid (Li et al., 1998; Luo et al., 1998); and for Bax, it is an unidentified Bax activating factor (probably Bid), and a mitochondrial outer membrane associating protein that are involved in the mitochondrial targeting event (Goping et al., 1998). These findings demonstrate that mitochondria play a pivotal role in apoptosis regulation in mammalian cells.

## *1.5 Mitochondria and Apoptosis*

The involvement of mitochondria in apoptosis was first suggested by the finding that the anti-apoptotic protein Bcl-2 is localized primarily to the mitochondrial outer membrane by its hydrophobic tail at the C-terminus, leaving the bulk of proteins facing the cytosol (Chao and Korsmeyer, 1998; Hockenbery et al., 1990; Nguyen et al., 1993). The C.

elegans Bcl-2 homologue Ced-9 is found in a bicistronic messenger RNA that encodes both Ced-9 and cytochrome b, implying a possible function and origin connection between Bcl-2 and mitochondria (Hengartner and Horvitz, 1994). Today, the mitochondrion is recognized as the sensor and executioner of apoptosis in mammalian cells. Although the exact mechanism(s) used by mitochondria to trigger and execute apoptosis is still unclear, several mechanisms have been proposed to explain the pivotal function mitochondria play in apoptosis. These include release of caspase activators such as cytochorome c and AIF; disruption of the electron transport chain, oxidative phosphorylation processes and ATP generation; and changes of cellular reduction-oxidation (redox) potential.

During apoptosis, cytochrome c is released from the mitochondrial intermembrane space into the cytosol where it can exert its function on caspase activation (Liu et al., 1996). Once translocated into cytosol, cytochrome c, in collaboration with Apaf-1, the mammalian homologue of the C. elegans death protein Ced-4, is able to activate procaspase-9 (Apaf-3) and other procaspases to trigger and/or enhance caspase activation cascades (Kluck et al., 1997; Li et al., 1997b; Yang et al., 1997). When cells are undergoing apoptosis, an apoptosis-inducing factor (AIF), a 50 kDa protein, is also released into the cytosol from the mitochondrial intermembrane space (Susin et al., 1996). AIF is capable of activating caspase-3 and inducing nuclear apoptosis in vitro (Susin et al., 1997; Susin et al., 1996). AIF activity can be blocked by the broad spectrum caspase inhibitor zVAD-fmk, suggesting that AIF itself may be another caspase. Some caspases such as caspase 3 are localized to mitochondria in certain cell types.

The mitochondrial inner membrane has a membrane potential($\Delta\psi$) with the intermembrane space side positively charged and matrix side negatively charged, and a proton concentration ($\Delta$pH) gradient where the intermembrane space side is acidic and the matrix space side is alkaline. Disruption of both $\Delta\psi$ and $\Delta$pH have been observed during apoptotic events (Castedo et al., 1996; Zamzami et al., 1995a; Zamzami et al., 1995b). A partially characterized mitochondrial permeability transition (PT) pore complex (PTPC) has been introduced to explain the mitochondria PT changes during apoptosis (Castedo et al., 1996; Zamzami et al., 1995a). The mitochondrial PTPC consists of protein components from both outer membrane and inner membrane. It may include the outer

membrane protein porin, also known as VDAC (voltage-dependent anion channel) and the benzodiazepine receptor, and the inner membrane protein adenine nucleotide translocator (ANT) and associated proteins such as cyclophilin D. The PTPC is a non-selective channel. When the PTPC is open, water and small molecules less than 1.5 kDa can move into the matrix through the pore. This process can cause mitochondrial swelling, and ultimately, the eruption of the outer membrane, leading to release of cytochrome c, AIF, and many other mitochondrial proteins into the cytosol.

Several signals can induce the mitochondrial permeability transition, including caspase activation, increased cytosolic $Ca^{2+}$ levels, mitochondrial targeting of Bax, Bad, Bid, and other pro-apoptotic Bcl-2 family proteins, nitric oxide, ceramide, $NAD(P)H_2$ and ATP depletion, $\psi$ disruption, increasing production of reactive oxygen species, and changes in cellular reduction-oxidation (redox) potential. Bcl-xL can inhibit mitochondrial permeability transition changes in cells treated with apoptotic stimuli (Vander et al., 1997). In many situations, the mitochondrial permeability transition is a self-amplifying process. It can lead to the collapse of the electron transport chain and oxidative phosphorylation.

The release of caspase-activating proteins from mitochondria into the cytosol could be a result of one or both of the following events: osmotic disequilibration and the opening of mitochondrial outer membrane channels. Osmotic disequilibrium can cause the opening of PTPC, a nonselective channel. The opening of the PTPC can lead to the equilibration of ions and respiratory substrates between the cytosol and mitochondrial matrix. This process can result in mitochondrial swelling, disruption of the mitochondrial $\psi$ and eventually the disruption of the mitochondrial membrane which results in the release of cytochrome c, AIF, and possibly other caspase-activating proteins into the cytosol. In cells where endogenous caspase inhibitors are present in large quantity, a large amount of cytochrome c must be released in order to activate the caspase cascade. This, however, results in the complete loss of function of the electron transport chain because of the loss of cytochrome c. The cell will die in a more necrotic manner because of the lack of ATP being generated. ATP may be required for the execution of the apoptotic process. Conversely, in cells that do not contain a high concentration of caspase inhibitors, only a small amount of cytochrome c disassociated from the electron transport chain will be sufficient to trigger the caspase-

activation cascade. In this case, the majority of cytochrome c can still associate with cytochrome b-c1 in the electron transport chain to generate ATP to facilitate late apoptotic events.

The mitochondrial outer membrane channels are poorly defined. There is evidence that the putative mitochondrial megachannel opens after the cell is challenged with apoptotic stimuli suggesting that cytochrome c and many other mitochondrial inner membrane proteins can go through the opened megachannel to the cytosol. Bcl-2 family members may have the capacity to form mitochondrial outer membrane ion conducting channels according to the crystal structure of Bcl-xL (Muchmore et al., 1996). The Bcl-2/Bcl-xL channel prefers cations while the Bax channel favors anions (Minn et al., 1997; Schendel et al., 1997). Bcl-2 can also prevent Bax from forming ion conducting channels under proper conditions (Antonsson et al., 1997; Schlesinger et al., 1997). In brief, Bcl-2 and Bax may form ion conducting channels in the mitochondrial membrane and thereby influence mitochondrial membrane permeability. Bax can induce caspase-independent apoptosis in a mitochondria dependent manner, suggesting that mitochondria have a fundamentally important role in Bax induced apoptosis in the cell. Bax can bind to the permeability transition pore complex (PTPC) of mitochondria (Marzo et al., 1998). The ANT (adenine nucleotide translocator) is a component of the PTPC. Atractyloside can induce mitochondrial membrane permeability changes and apoptosis by binding to the ANT. The PTPC purified from Bax-deficient mice could not be permeabilized in response to atractyloside (Marzo et al., 1998). Bax overexpression can induce cell death in wild-type but not in ANT-deficient yeast (Marzo et al., 1998). These data suggest that Bax exerts its pro-apoptotic function by cooperating with ANT within the PTPC to increase mitochondrial membrane permeability and to trigger cell death (Marzo et al., 1998).

## *1.6 Endoplasmic Reticulum and Apoptosis*

Although Bcl-2/Bcl-xL and other Bcl-2 family members are also localized to the ER, the importance of ER in apoptosis has not been extensively investigated. A search for Bcl-2 binding proteins by Far Western analysis has led to the identification of p28/Bap31, a 28

kDa polytopic integral membrane protein located in the ER, as a component of the Bcl-2 protein complex associated with the ER membrane (Ng et al., 1997). The N-terminal of Bap31 contains three transmembrane domains with the first and the third TM region carrying positively charged residues. The C-terminal domain of Bap31 contains a weak death effector homology domain, two caspase recognition sites, and an ER retention signal KKEE at the extreme C-terminus (Ng et al., 1997). Upon triggering of cell death, the two caspase recognition sites of Bap31 can be recognized by caspases and are cleaved into two proteins: p20 (N-terminal portion) and p10 (C-terminal) (Ng et al., 1997). When the p20 fragment is expressed in 293T cells, it can induce apoptosis (Ng et al., 1997). Purified caspase-8 but not caspase-3 can proteolytically cleave Bap31 at these two caspase sites to give p20 and p10 (Ng et al., 1997). In co-transfected 293T cells, Bap31, Bcl-2/Bcl-xL, procaspase-8, and Ced-4 form an ER membrane associated protein complex (Ng and Shore, 1998). Although the exact context of this complex in still elusive, Bap31 does not interact with procaspase-8 directly as judged by an in vitro GST fusion binding assay. Bap31 interacts with Ced-4 via the death effector homology domain of Bap31 in a GST fusion protein binding experiment (Ng and Shore, 1998). The interaction of Bap31 with Ced-4 contributes to the recruitment of procaspase-8 to the Bap31 complex (Ng and Shore, 1998). Bcl-xL weakly but directly interacts with the transmembrane region of Bap31. However, in 293T cells, the cytosolic region of p28/Bap31 has the ability to interact with Bcl-xL. This cooperative but indirect interaction between the Bcl-xL and Bap31 cytosolic regions relies on the presence of Ced-4 (Ng and Shore, 1998). A C-terminus deletion mutant of Ced-4 that has lost Bcl-xL binding ability fails to promote the association of Bcl-xL with Bap31, suggesting that an endogenous Ced-4 homologue is a key component of the Bap31 complex that bridges the interaction of Bap31 with Bcl-xL and procaspase-8 (Ng and Shore, 1998).

Although the function of ER localized Bap31, Bcl-xL, procaspase-8, and Ced-4-like protein complexes in apoptosis needs to be established, Bcl-xL can prevent the caspase processing of Bap31 (Ng et al., 1997). Co-expression of Bax can prevent Bcl-2 from associating with the Bap31 complex (Ng et al., 1997). This is reminiscent of the way Bax can prevent Bcl-xL from forming the mitochondrial procaspase-9/Apaf-1/Bcl-xL complex

and the way EGL-1 interrupts the CED-3/CED-4/CED-9 protein complex formation in *C.elegans* (Conradt and Horvitz, 1998).

Another ER localized protein, the inositol-1, 4, 5-trisphsophate receptor (IP3R-1), is a calcium channel located on the ER membrane, and may have an apoptotic regulatory function. Increases of intracellular $Ca^{2+}$ can induce apoptosis. Bcl-2 can block this apoptotic process by inhibiting the $Ca^{2+}$ efflux from the ER calcium channel, the IP3R-1. Cells which do not express the IP3R-1 are resistant to apoptosis and show no increase in intracellular $Ca^{2+}$ (Jayaraman and Marks, 1997). Given the fact that the Bap31 complex is ER localized, the Bcl-2 and Bap31 complexes at the ER membrane may exert their function directly or indirectly through regulation of the ER calcium channel.

The use of yeast complement screening to search for proteins that can inhibit Bax killing in yeast has led to the finding of another ER membrane protein, BI-1 (Bax inhibitor protein-1) (Xu and Reed, 1998). It may be involved in the regulation of $Ca^{2+}$ in the ER as well (Xu and Reed, 1998). However, its significance in apoptosis under physiological conditions needs to be established.

## *1.7 Cell Cycle and Apoptosis*

### 1.7.1 Introduction

Homeostasis is a dynamic process of cell proliferation, differentiation, and cell death. In order to sustain homeostasis, the balance between cell death and proliferation has to be strictly controlled. Apoptotic stimuli affect both cell proliferation and cell death. Over-expression of the anti-apoptotic protein Bcl-2 can cause neoplasia due to the inhibition of apoptosis., whereas, too much apoptosis in the neuronal system is the cause of many neuronal degenerative diseases. So, it is essential to monitor and eliminate cells that proliferate inappropriately. Apoptosis and proliferation are linked through cell cycle regulation. Many cell cycle regulatory proteins, such as the G1 regulator p53, pRb, and E2F, affect both cell division and cell death.

Cells have evolved sophisticated mechanisms to control their division. The activation of specific cyclin-cdk complexes at different cell cycle stages drives cells to progress to the next cycle stage. Cell cycle check points exist at different stages, including the G1/S boundary, S phase, and G2/M phases (Murray, 1994). They serve as safeguards to ensure that important cell cycle events of a particular phase are completed before entering the next phase (Murray, 1994). Mutation or deletion of genes that control cell cycle progression can increase the proliferation rate of cells. Inappropriate proliferation of cells can cause malignancy. Altered internal and external conditions can block the cell cycle progression at these checkpoints to ensure that no aberrant cells will be produced (Evan et al., 1992a). In many systems, the inappropriate activation or abnormal progression of the cell cycle can induce apoptosis (Evan et al., 1992a). One example is myc. Under normal cell cycle conditions, in the presence of growth factors, myc overexpression can prompt cell division and proliferation, while, in the absence of growth factors, myc overexpression will induce apoptosis (Thompson, 1998).

### 1.7.2 p53 and Cell Cycle Regulation

In more than 50% of human tumors, p53 is inactivated either by mutation, phosphorylation, or sequestration by viral proteins (Hollstein et al., 1994). p53 is involved in the control of the G1-to-S phase transition, in response to cell DNA damage (Baker et al., 1990; Diller et al., 1990; Fritsche et al., 1993; Kuerbitz et al., 1992). P53 levels can be dramatically increased through post-transcriptional mechanisms by a variety of upstream signals (Prives, 1998). P53 activation in the cell can block cell cycle progression at G1 phase. It may do this through several mechanisms, including the induction of cyclin-dependent kinase inhibitors like p21, p19ARF, and the DNA-damage induced protein kinases, such as ATM and DNA-PK. P53 activity is negatively regulated by a protein called Mdm2. Mdm2 can physically associate with p53 and target p53 to a degradation pathway or induce the phosphorylation and inactivation of p53. Both p53 and mdm2 are direct targets of kinases such as DNA-PK and ATM. The DNA-damage-induced phosphorylation of either p53 or mdm2 can block these two proteins from interacting, thus stabilizing and activating p53. The INK4a gene encodes two tumor suppressor proteins by altering its reading frame. The

first product p16ink4a, can bind to CDK4 and inhibit CDK4 from phosphorylating Rb, thus inhibiting cell cycle progression from the G1 to S-phase transition (Prives, 1998). The second product of INK4a encodes p19ARF. p19ARF can physically interact with mdm2 and block mdm2 from interacting with p53, therefore stabilizing p53 (de et al., 1998). Of note is that some protooncogenes such as SV40 large T antigen can induce a high level of p53 in the cell; however, this p53 is inactive, suggesting that the stabilization of p53 does not necessarily correlate with p53 activation. There is evidence suggesting that p53 activity may be regulated by its newly identified homologue p63 (Yang et al., 1998a). p63 can function as a dominant-negative regulator of p53 by physically interacting with p53 in tissues or cells which express both p63 and p53. This suggests that the homo-tetramerization of p53 is critical for its activation (Yang et al., 1998a). The adenovirus E1A oncogene has been reported to be able to activate and stabilize p53 through a signaling pathway that involves the Rb protein and the tumor suppressor p19ARF (Pomerantz et al., 1998). In ARF-null cells, the ability of E1A to activate p53 is severely impaired. The ARF-null cells are resistant to apoptotic stimuli as well. However, re-introduction of the p19ARF gene into ARF-null cells can induce p53 accumulation and resensitize ARF-null cells to apoptotic signals (Pomerantz et al., 1998).

### 1.7.3 p53 and Apoptosis

Many different signals can trigger p53-dependent apoptosis, and a single death trigger can initiate both p53-dependent and p53-independent pathways. For example, DNA damage, some anti-cancer drugs, and viral infections can induce both p53-dependent and p53-independent apoptosis. Therefore, p53 is involved in the induction of some, but not all forms of apoptosis.

p53 regulates the expression level of several proteins known to have important functions in the regulation of the apoptotic process. In a murine p53 temperature sensitive cell line, the Bcl-2 expression level is decreased and that of Bax is increased (Miyashita et al., 1994). Fas, a cell surface protein that triggers apoptosis upon FasL binding, is encoded by a target gene for transcriptional activation by p53 (Owen-Schaub et al., 1995). As of

today, the only known function of p53 is its transcription activation ability. However, the requirement of p53's transcriptional activation ability for apoptosis induction is controversial. If p53's transcriptional activation and repression functions are required for apoptosis induction, it may affect changes in the expression of known apoptotic regulatory genes such as Bcl-2, Bax and Fas.

### 1.7.4 Rb and Cell Cycle Regulation

The Rb, like p53, is also linked to cell-cycle regulation and is frequently inactivated in human diseases, including tumors of the retina, lung, breast, bladder, and prostate. Rb is also an early target protein of proteolytic degradation by caspase activation (Tan and Wang, 1998; Wang, 1997). The Rb gene product functions by binding to and inactivating members of the E2F family of transcription factors (Teodoro and Branton, 1997). Homozygous deletion of Rb is embryonic lethal in mice; the Rb-/- mouse embryos show increased apoptosis in neurons, liver, and hematopoietic precursors.

## *1.8 Caspase Activation via Death Receptors and Apoptosome*

Recent progress made on so-called death receptors has greatly expanded our understanding of the relations between signaling pathways and the caspase cascades that transmit and amplify apoptotic signals.

The death receptors are a growing subset of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily that shares an 80 amino acid conserved domain located in the cytoplasmic region known as the death domain (DD). Mammalian death receptors include Fas/APO-1/CD95 (Smith et al., 1994), TNFR1 (Smith et al., 1994), DR3/WSL-1/Apo-3/TRAMP/LARD (Chinnaiyan et al., 1996a), TRAIL receptors DR4/TRAIL-R1 (Pan et al., 1997), DR5/TRAIL-R2/Trick2/KILLER (Screaton et al., 1997), and DR6 (Pan et al., 1998a).

The death domain was also identified in several cytoplasmic proteins. Binding of death ligands, like TNF, FasL, and TRAIL to their respective death receptors can recruit a wide

variety of death domain-containing cytoplasmic adapter proteins to the death-inducing signaling complex (DISC). This recruitment is through the interactions of the death domain of receptor proteins and cytoplasmic death domain-containing adapter proteins. The adapter proteins that have been identified so far include FADD/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995), TRADD (Hsu et al., 1995), RIP (Stanger et al., 1995), RAIDD/CRADD (Duan and Dixit, 1997), and MADD (Schievella et al., 1997). The cytoplasmic DD-containing adapter proteins and large prodomain-containing procaspases such as procaspase-8 and procaspase-10 also contain another distinctive protein-protein interaction motif named the death effector domain (DED) whose structure is similar to another protein-protein interaction motif, CARD (caspase recruitment domain) which is present in some long prodomain caspases (Eberstadt et al., 1998).

All TNF family members including FasL form homotrimeric molecules. Crystal structure studies of the TNFR1 complex indicate that each TNF trimer binds three TNFR molecules. Because death domains also tend to associate with each other, the TNFR ligation leads to clustering of the receptor's death domains. The clustered death receptors can then recruit DD-containing adapter proteins from the cytosol into the death receptor complex. The recruited DD-containing adapter proteins can further interact with and activate down-stream procaspases such as caspase-8 and -10 through the interactions of their respective DEDs. For example, FADD/MORT1 depends on its DEDs to recruit the upstream pro-caspase-8 and/or pro-caspase-10 to the DISC. This recruitment process can result in pro-caspase autocatalytic activation. Caspase-8 autoactivation by association with the Fas DISC, can lead to the further amplification and activation of the caspases cascade (Medema et al., 1997). Downstream of the DISC, the Fas signaling pathway is involved in the activation of additional caspases such as caspase-1 (Enari et al., 1995), caspase-3 (Enari et al., 1996), caspase-4 (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), caspase-6 (Fernandes et al., 1995a) and caspase-7 (Fernandes et al., 1995b).

The important role that FADD plays in the death receptor signaling pathway is demonstrated by FADD knockout studies. FADD knockout mice are embryonic lethal (Yeh et al., 1998). FADD-/- mice cannot survive beyond day 11.5 of embryogenesis and show signs of cardiac failure, suggesting that the FADD signaling pathway may not function only

through death receptors, but may also have regulatory functions in the developmental process (Yeh et al., 1998). DR4, E1A, and c-myc can all induce apoptosis in FADD-deficient embryonic fibroblasts. But CD95, TNFR-1, and DR3 cannot, indicating that different death stimuli use different signaling pathways (Yeh et al., 1998).

Recently, two caspase activation complexes, the cell surface DISC and the mitochondrial apoptosome, have been located to the same caspase activation cascade. Fas/TNF induce the activation of caspase-8 via the DISC. The activated caspase-8 cannot only proteolytically cleave the downstream caspases but also can cleave Bid, a pro-apoptotic member of the Bcl-2 family of proteins (Luo et al., 1998). Bid normally resides in the cytosol. Upon cleavage by caspase-8, the truncated Bid can translocate to the mitochondrial outer membrane to induce the release of cytochrome c into the cytosol (Luo et al., 1998). The cytochrome c release can activate the apoptosome and lead to caspase-9 activation (Luo et al., 1998). The activation of caspase-9 can lead to another round of caspase amplification by activating the effector procaspase-3 (Hakem et al., 1998b). Caspase-9 is a very important cellular caspase whose deletion in mice is embryonic lethal, showing its role cannot be substituted by other caspases (Hakem et al., 1998b).

## *1.9 Protein Kinase Pathways and Apoptosis*

Apoptosis may be regulated through transcriptional activation mechanisms. At least two different mitogen-activated protein kinase (MAP kinase) cascades that converge to c-Jun N-terminal kinase (JNK , also known as SAPK or stress activated protein kinase) and p38/SAPK1 are implicated in the apoptosis process (Ichijo et al., 1997; Wang et al., 1997). These two pathways are preferentially activated in response to a variety of environmental stresses, such as UV light, X-ray, heat shock and growth factor withdrawal (Ichijo et al., 1997; Wang et al., 1997). JNK was first characterized as a MAP kinase family member which binds c-Jun and phosphorylates Ser63 and Ser73 located within the transactivation domain of c-Jun. c-Jun can heterodimerize with another transcription factor, Fos, to form the AP-1 transcription factor which can regulate the transcription of many genes. In many instances, the p38/SAPK2 and JNK/SAPK1 pathway activation induce apoptosis. However,

the response of mammalian cell protein kinase pathways to death signals is complex. It often involves multiple signaling pathways that act in concert to influence cell fate.

## *1.10 Proteases and Apoptosis*

### 1.10.1 The Caspase Family

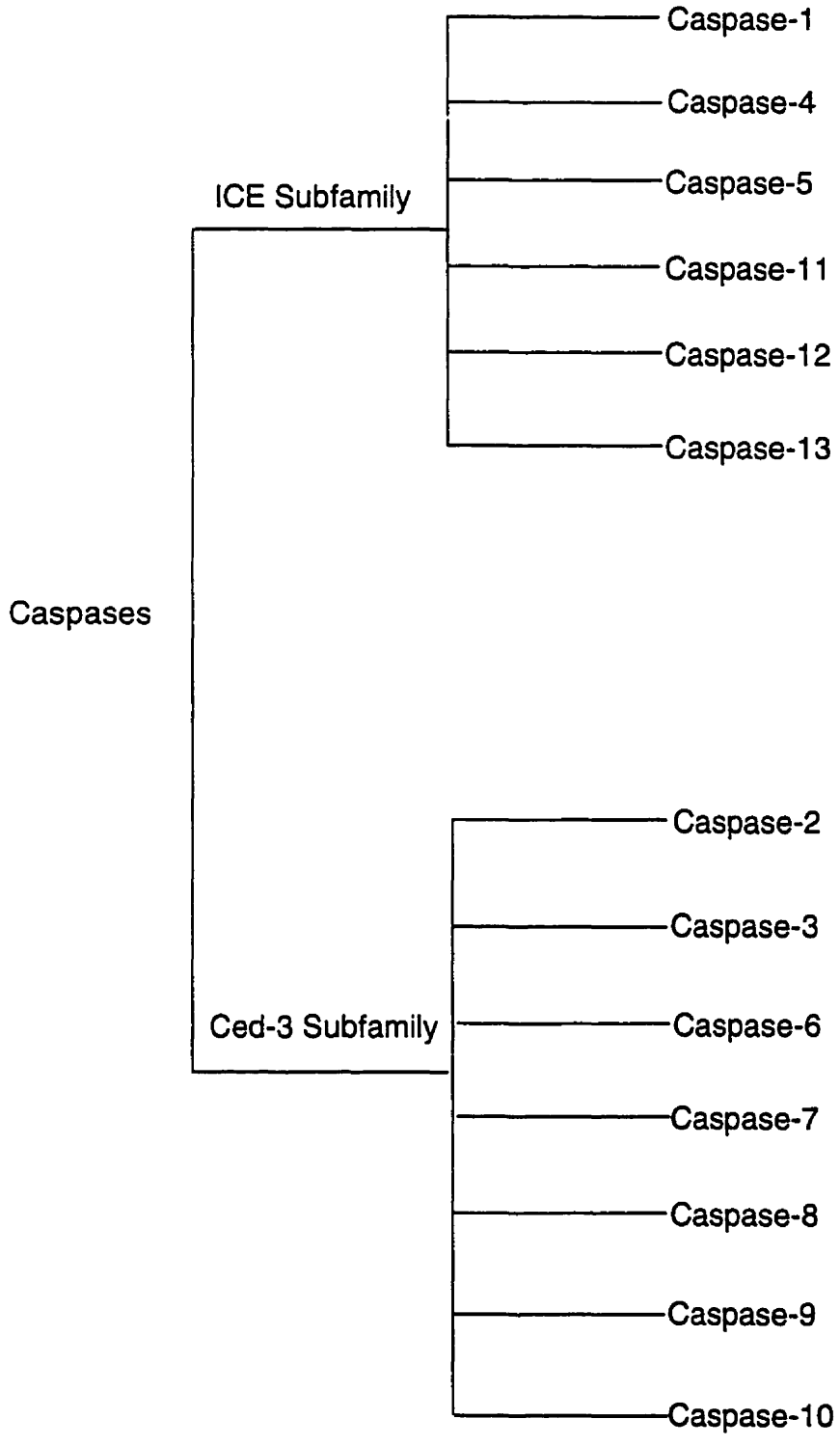
Since the discovery of the interleukin 1-converting enzyme (ICE) (Thornberry et al., 1992), the first member of a growing family of cysteine proteases now named caspases (cysteinyl asparatate protease) (Alnemri et al., 1996), twelve other members have been reported (see Figure 1-3 on page 39). They are caspase-2 (Ich-1/Nedd-2 (Kumar et al., 1994; Wang et al., 1994b), caspase-3 (apopain, CPP32, Yama) (Fernandes et al., 1994; Nicholson et al., 1995; Tewari et al., 1995), caspase-4 (ICErel -II, TX, ICH-2) (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), caspase-5 (ICErel -III, TY) (Munday et al., 1995), caspase-6 (Mch2) (Fernandes et al., 1995a), caspase-7 (Mch3, ICE-LAP3, CMH-1) (Duan et al., 1996a; Fernandes et al., 1995b; Lippke et al., 1996), caspase-8 (FLICE, MACH, Mch5) (Boldin et al., 1996; Fernandes et al., 1996; Muzio et al., 1996), caspase-9 (ICE-LAP6, Mch6) (Duan et al., 1996b; Li et al., 1997b; Srinivasula et al., 1996b), caspase-10 (Mch4 ) (Fernandes et al., 1996; Vincenz and Dixit, 1997), caspase-11(mICH-3, mCASP-11) (Van et al., 1997; Wang et al., 1996d), caspase-12 ( mICH-4 ) (Van et al., 1997), and caspase-13 (ERICE) (Humke et al., 1998).

### 1.10.2 Features of the Caspase Family

All members of the caspase family share several amino acid residues important for substrate binding and catalysis (Cryns and Yuan, 1998; Thornberry et al., 1992; Walker et al., 1994). They are all synthesized as catalytically inactive zymogens that can be activated by proteolytic cleavage after specific Asp residues in the interdomain linker regions. The caspase family of proteases contains a QACXG pentapeptide. The cysteine in this pentapeptide sequence participates in the catalytic reaction (Thornberry et al., 1992).

Figure 1-3 The caspase family.

# Functional Classification of Caspases



Caspases exist in the cytosol as a single polypeptide zymogen consisting of a prodomain of variable length plus a large subunit, which contains the catalytic cysteine, and a small subunit (Cryns and Yuan, 1998; Nicholson and Thornberry, 1997). Two subunits flanked by Asp-X sites suggest that proteolytic cleavage activation is mediated through an aspartate-specific protease. All caspases require accurate proteolytic processing at the internal aspartate residues to generate an active enzyme. This proteolytic processing can be accomplished by other proteases, often other caspases, or by autocatalytic cleavage (Takahashi and and Earnshaw, 1996). The mature active caspase is a tetramer consisting of two heterodimers formed by the large and small subunits, according to the crystal structure analysis of caspase-1 and -3.

### 1.10.3 Classification of Caspases

All caspases require an aspartic acid residue in the substrate P1 position and cleave the substrate after the P1 Asp residue (Thornberry et al., 1992). Although the mechanism of the action is the same and conserved for all caspases, each caspase has a different substrate specificity. Based on their substrate specificity, the caspases are divided into three subfamilies by positional substrate scanning studies (Thornberry et al., 1997b). The most variable amino acid residue is found at substrate position P4. Caspases-2, -3, and -7, and Ced-3 have a selection preference for the DExD motif, and caspases-6, -8, -9, -10 and granzyme B favor the motif (I/V/L)ExD. These two subfamilies of caspases together are known as the Ced-3 family. The third subfamily of caspases is also called the ICE-family. It consists of caspases -1, -4, -5, -11, -12, and –13. The ICE-family prefers the WEHD substrate sequence.

The members of the Ced-3 family of caspases are involved in apoptosis. The main function of the ICE-subfamily of caspases is in cytokine maturation and inflammation. Interestingly, the Ced-3 subfamily of caspases do not contain hydrophobic amino acids in the P4 substrate position (Thornberry et al., 1997b).

The caspases can also be divided into two subgroups, the initiator caspases and the effector caspases, based on their structure and function in apoptosis. Structurally, all the

initiator caspases possess a large N-terminal prodomain, while the effector caspases contain a short N-terminal prodomain. The initiator caspases include Ced-3, caspase-1, -2, -4, -5, -8, -9, and -10. The effector caspases are caspase-3, -6, -7, -11, -12, and -13. The current view about caspase activation in the cell is that the initiator caspases are first proteolytically activated; the active initiator caspases can then cleave and activate the downstream effector caspases to start the caspases activation cascade. It is the active effector caspases that specifically cleave and destroy the cellular substrates and result in irreversible cell death. In vitro experiments demonstrate that the substrate cleavage preferences of caspase-8, -9, and -10 match the cleavage sites in several effector caspases such as caspase-3 and -7. This suggests that these upstream initiator caspases directly activate their downstream effector caspases. The substrate cleavage sites of effector caspases such as caspase-3 closely resemble the cleavage sites in the majority of known caspase substrates . This implies that caspase-3 has important roles in the proteolytic degradation of cellular targeting proteins (Boldin et al., 1996; Muzio et al., 1996; Muzio et al., 1997; Orth et al., 1996; Srinivasula et al., 1996a; Thornberry et al., 1997a) .

## 1.10.4 CARD and DED, the Protein-protein Interaction Motifs

The long prodomains of caspase-8 and caspase-10 each contain two copies of the death effector domain (DED). The long prodomains of Ced-3, caspase-1, -2, -4, and -9 all contain a caspase recruitment domain (CARD). RAIDD/CRADD, Ced-4, and Apaf-1 also contain a copy of CARD. NMR structure and mutagenesis studies show that the CARD and DED are structurally similar despite the divergence of amino acid sequence between the two, suggesting that DED and CARD function by mediating protein-protein interactions (Eberstadt et al., 1998).

Caspase-8 is one of the initiating caspases of the apoptotic cascade that is activated by engagement of death receptors belonging to the Fas or tumor necrosis factor receptor (TNFR) family (Boldin et al., 1996; Muzio et al., 1996). In brief, the long prodomain of caspase-8 contains two copies of a death effector domain (DED) which is responsible for its recruitment to the Fas or TNF death-inducing signaling complex (DISC) through

interacting with the corresponding DED domains of Fas-associated protein with death domain (FADD). The recruitment of initiator caspase-8 to DISC can result in its proteolytic activation through the removal of the prodomain and cleavage at the internal aspartic acid residues to give large and small subunits. The active caspase-8 then can cleave downstream effector procaspases such as caspase-3, and pro-apoptotic Bcl-2 family members like Bid, to further amplify the caspase cascade (Boldin et al., 1996; Luo et al., 1998; Muzio et al., 1996).

### 1.10.5 Knockout Studies of Caspases

Several caspases, including caspase-1, -2, -3, -9, and –11, have been genetically deleted in mice. Except for caspase-9, caspase-8, and caspase-3, mice deficient in all other caspases are viable and normal, or in some cases, exhibit minor cell death abnormalities. These studies show that caspase-9 and caspase-3 have a non-redundant role in development. It also shows that other mammalian caspases, such as caspase-1, -2, and -11, may have redundant functions, or serve as an amplification step in the caspase pathway, or have tissue and cell type specificity.

It has been proposed that cytochrome c initiates apoptosis by inducing the formation of the caspase-9/Apaf-1 complex (Li et al., 1997b). The physical interaction between caspase-9 and Apaf-1 is mediated through their CARD (caspase recruitment domain). This interaction is critical for the auto proteolytic activation of caspase-9 (Srinivasula et al., 1998). Bcl-xL can interact with Apaf-1 oligomers and prevent caspase-9 from being activated (Srinivasula et al., 1998).

Caspase-9 gene deletion in mice embryos causes perturbation of brain structure especially with the cortex and forebrain because of more neurons growing in the absence of apoptosis (Hakem et al., 1998a). It demonstrates that caspase-9 is essential for apoptosis in neuronal cells (Hakem et al., 1998a). This is also the first demonstration that caspase-9 is required for caspase-3 activation since caspase-3 cannot be activated by cytochrome c release in caspase 9-/- mouse (Hakem et al., 1998a).

### 1.10.6 Caspase Substrates

Numerous caspase family substrates have been identified (see Figure 1-4 on page 44). Figure 1-4 is a list of some of these caspase substrates.

### 1.10.7 Peptide-derived Inhibitors of Caspases

Many peptide caspase inhibitors have been constructed based upon the presumed substrate specificity of the different caspases. The first caspase-1 subfamily specific inhibitor, YVAD-fmk (acetyl-Tyr-Val-Ala-Asp-fluoromethylketone), was based upon the tetrapeptide recognition sequence YVAD present in prointerleukin-1β, a natural substrate for caspase-1. Similarly, a tetrapeptide inhibitor, DEVD-fmk (acetyl Asp-Glu-Val-Asp-fluoromethylketone), was designed around the putative PARP cleavage site, DEVD, to be selective for the CED-3 subfamily of caspases (Lazebnik et al., 1994). ZVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) is a broad-spectrum caspase inhibitor that inhibits all known caspases. Aldehyde derivatives of these inhibitors are reversible inhibitors and form a thiohemiacetal with the active site cysteine in the caspase, whereas chloromethyl, fluoromethyl, acyloxymethyl, apyrazoloxymethyl, and phosphinyloxymethyl ketones are irreversible since they covalently form a thiomethyl ketone with the sulfur atom of the active site cysteine. In vitro data show that Ac-YVAD-aldehyde is a potent inhibitor of caspase-4 and caspase-1 (The ICE subfamily) but is ineffective for caspase-3 and caspase-7. In contrast, caspase-3 and caspase-7 are potently inhibited by Ac-DEVD-CHO (Margolin et al., 1997).

## *1.11 Granzyme B and Apoptosis*

Cytotoxic T lymphocytes recognize foreign viral antigens in association with class I MHC glycoproteins on the surface of any host cell. CTL and NK-mediated cytotoxicity and apoptosis can be a result of either granule exocytosis or the Fas-dependent pathway (Kagi et al., 1994; Kojima et al., 1994; Walsh et al., 1994). The granule initiated cell killing requires two molecules, the lymphocyte-specific granule serine esterase Granzyme B and the pore-

Figure 1-4 Caspase substrates.

## Caspase substrates whose function in apoptosis have been defined

Bcl-2 (Cheng et al. 1997)
Bcl-xL (Clem et al. 1998)
DFF-45/ICAD (Liu et al. 1997; Enari et al. 1998; Sakahira et al. 1998)
p28 Bap31 (Ng et al. 1997)
PKC δ and υ (Emoto et al. 1995; Datta et al. 1997)
MEKK-1 (Cardone et al. 1997)
PAK2/hPAK65 (Rudel and Bokoch 1997; Lee et al. 1997)
pro-caspases (for review, Fraser and Evan 1996; Nicholson and Thornberry 1997)
nuclear lamins (Lazebnik et al. 1995; Rao et al. 1996;
Takahashi et al. 1996)
Gas2 (Brancolini et al. 1995)
gelsolin (Kothakota et al. 1997)

## Caspase substrates which have been functionally linked to apoptosis

PARP (Lazebnik et al. 1994)
DNA–PKCS (Casciola-Rosen et al. 1995; Han et al. 1996; Song et al. 1996)
U1–70kD (Casciola-Rosen et al. 1994, 1996)
hnRP C1 and C2 (Waterhouse et al. 1996)
DSEB/RF-C140 (Ubeda and Habener 1997; Rhe´ aume et al. 1997)
Sp1 (Piedrafita and Pfahl 1997)
fodrin (Martin et al. 1995a; Greidinger et al. 1996; Cryns et al. 1996; Vanags et al. 1996)
actin (Mashima et al. 1995; Kayalar et al. 1996)
keratins (Caulin et al. 1997; Ku et al. 1997)
FAK (Wen et al. 1997)
β-catenin (Brancolini et al. 1997)
D4-GDI (Na et al. 1996)
RB (Janicke et al. 1996; Tan et al. 1997)
PITSLRE kinase (Lahti et al. 1995; Beyaert et al. 1997)
PRK2 (Cryns et al. 1997)
phospholipase A2 (Wissing et al. 1997)
IkB-α (Barkett et al. 1997)
rabaptin-5 (Cosulich et al. 1997)
MDM2 (Chen et al. 1997; Erhardt et al. 1997)
Huntingtin (Goldberg et al. 1996)
presenilins 1 and 2 (Kim et al. 1997a; Loetscher et al. 1997; Vito et al. 1997)
DRPLA (Miyashita et al. 1997)
SREBPs (Wang et al. 1996)

forming protein perforin (Kagi et al., 1994; Kojima et al., 1994; Walsh et al., 1994). Perforin can form nonspecific ion channels on the plasma membrane in the presence of calcium. However, expression of perforin itself without Granzyme B cannot induce apoptosis (Kraut et al., 1992). Similarly, purified Granzyme B alone also fails to induce apoptosis (Kraut et al., 1992). Both perforin and Granzyme B are required to trigger apoptosis, although the exact mechanism by which these two proteins interact to mediate cell death is still unknown (Kraut et al., 1992).

Granzyme B is the only non-caspase known to cleave and activate multiple caspase members involved in apoptosis (Shi et al., 1992). Granzyme B is an aspartate-specific serine protease employed by cytotoxic T cells to kill target cells (Martin and Green, 1995). The caspases that can be cleaved and activated by Granzyme B include caspase-1 (Duan et al., 1996b), caspase-3 (Fernandes et al., 1996), caspase-7 (Fernandes et al., 1996), caspase-8 (Boldin et al., 1996), caspase-10 (Fernandes et al., 1996), and caspase-11 (Wang et al., 1996d). Analysis of the cleavage sites in caspase-3, -7, and -10 indicate that Granzyme B preferentially cleaves at the (I/V/L) XXD sequence located at the C-terminal end of the large subunit of the ICE subfamily of procaspases (Martin et al., 1996). Initial cleavage at this site is essential for productive processing of caspase zymogens, suggesting that they are required for Granzyme B and perforin-induced apoptosis (Martin et al., 1996; Shi et al., 1996). Inhibition of the ICE subfamily of caspases using tetrapeptide inhibitors such as Ac-DEVD-CHO or Ac-YVAD-CHO, can impair the Granzyme B induced apoptosis (Shi et al., 1996). A dominant negative form of ICE can suppress apoptosis induced by Granzyme B. Mouse fibroblasts devoid of ICE are resistant to apoptosis mediated by Granzyme B, suggesting that Granzyme B functions via the ICE subfamily of caspases (Shi et al., 1996).

Granzyme B resides in the cytosol after crossing the target cell plasma membrane. Granzyme B is also translocated to the nucleus, although the functional significance of nuclear Granzyme B is still unclear (Trapani et al., 1996). Granzyme B can enter the cell cytoplasm in an energy-dependent manner without the need for perforin (Shi et al., 1997). Granzyme B's ability to initiate apoptosis and localize to the nucleus depends on perforin (Shi et al., 1992; Shi et al., 1997).

## *1.12 Ceramide and Apoptosis*

Ceramide is a sphingosine-based lipid signaling molecule that can mediate cell cycle and apoptosis (Pushkareva et al., 1995; Spiegel et al., 1998; Spiegel et al., 1996). Ceramide is one of the most hydrophobic molecules in mammalian cells, and it therefore tends to remain within the membrane lipid bilayer. Cellular ceramide is generated from de novo synthesis or from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). There are two forms of SMase in cells, neutral SMase and acid SMase. The activation of neutral SMase is involved in the extracellular signal regulated kinase (ERK) cascade and inflammatory responses. Acid SMase activation in response to TNF can induce the stress-activated protein kinase/C-Jun kinase (SAPK/JNK) cascade and apoptosis (Haimovitz-Friedman et al., 1997; Obeid et al., 1993; Pushkareva et al., 1995). Many environmental stresses act directly on the cell membrane to activate the acid dependent sphingomyelinase and hydrolyze sphingomyelin into ceramide and choline phosphate (Haimovitz-Friedman et al., 1997; Jarvis et al., 1994; Obeid et al., 1993). The effect of ceramide on apoptosis is specific because naturally occurring dihydroceramide has no effect on apoptosis. The exact mechanism by which ceramide enhances apoptosis is not clear. It has been reported that the mitochondrial permeability transition (PT) is a commitment step in ceramide signaling to apoptosis and that Bcl-2 can effectively inhibit ceramide induced mitochondrial PT changes and release of apoptotic inducing factor (AIF) (France-Lanord et al., 1997; Obeid et al., 1993; Susin et al., 1997). Deletion of the death domain from the TNF receptor, as well as overexpression of dominant negative FADD/MORT1, can block TNF induced ceramide generation and apoptosis (Chinnaiyan et al., 1996b). Synthetic or purified ceramide can bypass the anti-apoptotic effect of a dominant-negative FADD/MORT1 mutant and restore apoptosis, indicating that ceramide generation is sufficient for downstream activation of the death receptor complex and functions in a Bcl-2 inhibitable fashion (Chinnaiyan et al., 1996b). Ceramide can regulate a variety of transcription regulators including c-Fos, Rb, c-myc, c-jun, and others. Mouse deficient in an acid SMase fail to undergo ionizing radiation-induced apoptosis, suggesting that ceramide functions as a "sensor" of apoptosis rather than a "executioner" (Ariga et al., 1998; Haimovitz-Friedman et al., 1997; Jarvis et al., 1994; Obeid et al., 1993; Spiegel et al., 1998).

While ceramide is an important regulatory component of apoptosis induced by TNF, FasL, and other death stimuli, sphingosine-1-phosphate (SPP), a further metabolite of ceramide, may be a second messenger involved in promoting cell proliferation and survival (Cuvillier et al., 1998; Spiegel et al., 1998). SPP protects cells from apoptosis by inhibiting the ceramide increase in death triggered cells (Spiegel et al., 1998). Thus the dynamic balance between levels of sphingolipid metabolites, ceramide, and SPP, and subsequent regulation of kinases such as JNK and ERK, are the key factors that determine whether the cell should survive or will die (Spiegel et al., 1998).

## *1.13 Viral Proteins Interact with Host Apoptotic Machinery*

### 1.13.1 Introduction

Apoptosis is used as a defense mechanism by the host cell to eliminate virus-infected cells. The host prevents viruses from completing their replication cycle and producing viral progeny, thereby preventing further infection of neighboring cells (Rudin and Thompson, 1997). However, viruses have evolved mechanisms to overcome these host defenses (Teodoro and Branton, 1997). Some viruses encode genes that can inhibit apoptosis of infected cells (Teodoro and Branton, 1997), while for others, apoptosis is an essential process in the late phase of infection. It facilitates the release of the viral progeny and the infection of neighboring cells, without causing host inflammation responses. Many virally encoded proteins can interact with cellular components of the apoptotic pathway and modulate apoptosis to favor virus production.

Viruses use several mechanisms to inhibit host cell apoptosis. Some viruses encode a homologue of the anti-apoptotic protein Bcl-2 to inhibit apoptosis in the host. Adenovirus E1B 19kDa (Rao et al., 1992), african swine fever virus LMW5-HL (Neilan et al., 1993), and human herpesvirus 8 Ksbcl-2 (Russo et al., 1996), are all viral homologues of Bcl-2 which function to prevent premature cell death caused by viral infection. A number of viruses also encode caspase inhibitors as a means of blocking apoptosis. Among these viral caspase inhibitors are the cowpox virus cytokine response modifier A (CrmA) (Antoku et al.,

1997), a baculovirus protein named p35, an inhibitor of apoptosis protein (IAP) (Bertin et al., 1996; Birnbaum et al., 1994), and herpesvirus FLICE inhibitory protein (FLIP) (Thome et al., 1997). Cowpox virus (CrmA) is a 38-kDa serine protease inhibitor. It can effectively block caspase-8 mediated processing of effector caspases, apparently by forming a stable complex with the caspase (Srinivasula et al., 1996a; Zou et al., 1997). The p35 protein encoded by baculovirus was discovered as a viral gene mutation that causes premature death of the host cells (Bertin et al., 1996) (242). The poxvirus CrmA inhibits caspase-1 and -8 most effectively, whereas the baculovirus protein p35 inhibits numerous activated caspases.

### 1.13.2 FLIPS (FLICE Inhibitory Proteins)

Fas and TNF-mediated apoptosis pathways involve receptors, adapters, and proteases. As discussed earlier, the protein-protein interaction via DED domains between adapters and procaspase-8 play fundamental roles for this caspase activation pathway, and have proven to be excellent targets for viruses. Several viral proteins contain DED-related motifs; these include the human herpesvirus-8 K13(HHV-8), the equine herpesvirus type 2 E8 protein, the bovine herpesvirus-4 protein E1.1, the herpesvirus saimiri (HVS) orf71, and the human molluscipox virus mc159/mc160. These viral proteins can interact with FADD and/or the caspase 8 (FLICE) pro-domain when cotransfected into mammalian cells and are termed FLIPs (FLICE-inhibitory proteins). Human herpesvirus FLIPs interact with the Fas-Associated Death Domain Protein (FADD) and block Fas and TNF-associated DISC formation and subsequent caspase-8 activation (Thome et al., 1997).

### 1.13.3 Viral and Mammalian Inhibitors of Apoptosis (IAPs)

The inhibitors of apoptosis proteins (IAPs) are a family of anti-apoptotic proteins. The baculovirus IAPs were the first members of this family be identified based on their ability to complement a p35 mutant virus (Clem et al., 1991).

Homologues of baculovirus IAP have also been reported in eukaryotes. D-IAP1, a Drosophila IAP homologue, was identified in a screen for mutations which enhance the

effect of RPR (Reaper gene product) induced apoptosis in Drosophila developing eyes. D-IAP inhibits reaper-mediated apoptosis in Drosophila, possibly by interaction with the DCP-1/drICE protease which has been defined as a caspase (Hay et al., 1995). A second Drosophila IAP, D-IAP2 was identified by a search of the databases for sequences homologous to the known IAPs (Harvey et al., 1997). The first human IAP to be identified, NAIP (the neuronal apoptosis inhibitory protein), was isolated based on its function in the neurodegenerative disorder, SMA (spinal muscular atrophy) (Roy et al., 1995). In many SMA patients, the NIAP gene is deleted. This is in agreement with the current hypothesis that SMA is caused by the failure to inhibit apoptosis. Subsequently, four human IAPs have been identified. They are c-IAP-1/HIAP-2/hMIHB (Duckett et al., 1996), c-IAP-2/HIAP-1/hMIHC (Roy et al., 1995), X-IAP/hILP (Deveraux et al., 1997), survivin (Ambrosini et al., 1997). Mammalian iap gene homologues, c-IAP-1 and c-IAP-2, interact with TNFR2 via the TRAF1 and TRAF2 (TNF receptor associated factor 1 and 2) adapter proteins and effectively suppress apoptosis, suggesting they may function by stimulating survival pathways.

A common structural feature of all IAP family members is a motif termed the baculovirus IAP repeat (BIR) that is present in either two or three copies (Birnbaum et al., 1994). The baculoviral IAPs and the Drosophila IAPs contain two BIR domains, thereas three of the human family members and a second Drosophila IAP contain three such domains. Survivin, in contrast, contains only one BIR domain, implying that a single BIR can be sufficient for the anti-apoptotic activity. With the exception of NAIP and survivin, all other known IAP family members also contain a RING finger domain at their carboxyl-terminus whose exact function remains elusive.

Recent studies of the human X-chromosome-linked iap gene product (XIAP) have shown that it functions by directly inhibiting at least two members of the caspase family of proteases (Deveraux et al., 1997). XIAP was found to effectively inhibit many cell death signals in vitro, including cytochrome c mediated apoptosis and is not affected by exogenously added Bcl-2. XIAP functions by directly binding to the partially processed products of the caspase-3 and-7 zymogens. In the presence of XIAP, the caspase-3 precursor was cleaved at a site between the large and small subunits without the subsequent

removal of the pro-domain. Thus caspase-8 can be activated in the presence of XIAP, but the subsequent activation of caspase-3 and other downstream caspases is inhibited. In addition to the ability of XIAP to inhibit caspase-mediated death in vivo, XIAP was shown to efficiently bind and inhibit caspase-3 in vitro (Deveraux et al., 1997). Interestingly, although XIAP could bind and inhibit a partially processed caspase in vitro, it could not bind the unprocessed pro-zymogen form of the caspase, suggesting that XIAP binds only to active caspase-3 but does not bind its inactive precursor.

## 1.13.4 Adenovirus Proteins Interact with Host Apoptotic Machinery

### 1.13.4.1 Introduction

The target cells of adenovirus infection are usually nondividing epithelial cells. In order to successfully replicate, adenoviruses carry a transcription activator, the early region 1A (E1A) protein, to drive infected cells into S-phase and facilitate viral DNA replication. The unexpected entrance into S-phase caused by E1A, however, can trigger apoptosis in these terminally differentiated host cells.

### 1.13.4.2 E1A Proteins

E1A is the first viral transcription unit to be expressed after the viral infection. Two early mRNAs transcribed from E1A region encode two proteins named 12S and 13S according to their sedimentation coefficients. The sequences of 12S and 13S are identical except for an additional 46 amino acid region that is missing in the 12S E1A. Both the 12S (243R) and 13S (289R) E1A contain a nuclear targeting sequence (Dyson et al., 1992; Flint and Shenk, 1997; Moran and Mathews, 1987; Nevins, 1995). This nuclear targeting sequence may be important for the transcriptional regulation and host DNA synthesis stimulation functions of E1A. Three major conserved regions, CR1, CR2, and CR3 are present in the 13S product while 12S E1A contains only CR1 and CR2 (Dyson et al., 1992; Flint and Shenk, 1997; Moran and Mathews, 1987; Nevins, 1995). CR1 and CR2 are critical regions for E1A to interact with Rb, p107, p130, p300, and other cell cycle regulator

proteins (Teodoro and Branton, 1997). CR3 is involved in 13S E1A transcription activation. The 13S E1A product can indirectly interact with DNA because the CR3 region binds to a number of transcription factors that can bring E1A to promoter regions of DNA. Host DNA synthesis is under the control of E2F, an important transcription activator that enhances the expression of a variety of important proteins which regulate DNA synthesis. Rb functions by binding to E2F and preventing E2F from performing its trans-activation function. E1A apparently competes with E2F for the same binding site on Rb, thereby freeing E2F to trans-activate a variety of promoters involved in cell cycle regulation, DNA synthesis, and cell death (Flemington et al., 1993; Helin et al., 1993). E1A expression can be oncogenic. This may be the result of its ability to induce DNA synthesis and transcription activation of genes involved in cell growth or proliferation.

Adenoviruses have evolved several strategies to counteract the host apoptosis process during the early stages of viral infection. This is largely carried out by two viral early region B (E1B) products, the E1B 55K and E1B 19K proteins (Rao et al., 1992; White and Cipriani, 1990).

#### 1.13.4.3 RID (Receptor Internalization and Degradation)

The adenovirus RID (for Receptor Internalization and Degradation) protein complex can mediate the internalization and destruction of the cell surface death receptor Fas. Forced degradation of Fas can result in apoptosis resistance in adenovirus infected cells, another mechanism that adenovirus utilize to escape the host immunoresponse and apoptosis defenses (Tollefson et al., 1998).

#### 1.13.4.4 E1B 55kDa

The adenovirus E1B transcript encodes both E1B 19kDa and 55kDa proteins from the same transcript using different reading frames; thus there is no sequence homology between the two E1B proteins. One of the major functions of E1B 55K is to bind to the N-terminal acidic region of p53, which is responsible for its transactivation activity, inactivate

p53-mediated transcription and hence inhibit p53 dependent apoptosis and cell cycle arrest (Teodoro and Branton, 1997).

### 1.13.4.5 E1B 19kDa

The second protein encoded by the E1B region is E1B 19kDa. It is an anti-apoptotic protein, but acts differently than E1B 55kDa. E1B 55kDa can block both p53- dependent and -independent apoptosis. E1B 19kDa is a viral functional homologue of cellular Bcl-2. It is believed that E1B 19KDa inhibits the apoptotic pathway through a mechanism similar to that of Bcl-2. The ability of E1A to cooperate with E1B 19kDa to transform BRK (Baby Rat Kidney) cells is well documented. Bcl-2 can substitute for E1B 19KDa in this cellular transformation assay suggesting the anti-apoptotic functions of E1B 19K and Bcl-2 are interchangeable under certain conditions.

Infection of human cells by an E1B 19kDa mutant adenovirus yields a phenotype characterized by DNA fragmentation and many other hallmarks of apoptosis, as well as poor virus yields. A class of adenovirus type 2 mutants yield large plaques (lp) on infected monolayers of cells. This phenotype has been physically linked within the E1B 19kDa coding region (Kumai et al., 1989). Subsequent studies have found that several adenoviruses including Ad2, Ad5, and Ad12 mutants carrying defects in the E1B 19kDa coding region also give large plaques. These mutants were named 'cyt/deg' mutants because they induce extensive cytolysis (cyt) and fragmentation of cellular and viral DNA (deg) in infected cells.

### 1.13.4.6 Proteins Interact with E1B 19kDa

E1B 19kDa does not show significant overall sequence homology to other Bcl-2 family members. However, two regions of E1B 19kDa show sequence similarities to the conserved BH1 and BH3 domains of Bcl-2. Seven cellular proteins that interact with E1B 19kDa have been identified. There are Nip1, Nip2, Nip3, Bax, Bak, Bik, and nuclear lamin (Boyd et al., 1994; Farrow et al., 1995; Han et al., 1996; Rao et al., 1996). Except nuclear lamin, all six proteins also interact with Bcl-2 or Bcl-xL, suggesting that E1B 19kDa and Bcl-2 use a

common apoptotic regulatory pathway. Also, Ced-4 can interact with E1B 19kDa in a yeast two-hybrid system (Han et al., 1998).

### 1.13.4.7 Function and Mechanism of E1B 19kDa

E1B 19kDa is modified by both fatty acylation and phosphorylation (McGlade et al., 1989). It is associated with nuclear and cytoplasmic membranes, intermediate filaments of the cytoplasm and nuclear lamins (Rao et al., 1996). E1B 19kDa is implicated in the mobilization of $Ca^{2+}$ ions. The level of intracellular $Ca^{2+}$ ion appears to be an important regulator of cell death. Cells deficient in the type 1 inositol-1, 4, 5-triphosphate receptor (IP3R-1), a calcium release channel located on the ER membrane, fail to respond to a variety of apoptotic stimuli. This is due to an inability to raise intracellular calcium ion concentration (Jayaraman and Marks, 1997). E1B 19kDa can also sequester FLICE from being recruited to the DISC when Fas is stimulated, and can inhibit FADD-induced apoptosis. These results suggest that E1B 19kDa may block Fas-induced apoptosis downstream of FADD recruitment of FLICE but upstream of FLICE activation (Perez and White, 1998). The ability of E1B 19kDa to sequester FLICE and inhibit apoptosis triggered by the death receptor complex signaling is inconsistent with the observation that E1B 19kDa can effectively block Fas and TNF induced apoptosis, while Bcl-2/Bcl-xL cannot very efficiently. The binding of E1B 19kDa to nuclear lamin can slow down the degradation of lamin and many other caspase-targeting nuclear proteins, and slow down the nuclear apoptotic phenotype (Rao et al., 1996). E1B 19kDa can also modulate transcriptional activities by enhancing the JNK/SAPK1 signaling pathway and induce c-Jun dependent transcription (See and Shi, 1998). Whether this transcription activation ability is related to E1B 19kDa's anti-apoptotic function is not clear. More recently, E1B 19Da was found to associate with Ced-4. This interaction is required for the inhibition of FLICE-mediated apoptosis, suggesting that E1B 19kDa functions the same way as Bcl-xL by interacting with ced-4 and Apaf-1 and preventing them from activating the caspases (Han et al., 1998).

#### 1.13.4.8 ADP (Adenovirus Death Protein)

The E3-11.6K (Adenovirus Death Protein, ADP) is responsible for the death of host cells at the end of viral replication events (Tollefson et al., 1996). E3-11.6 mutant adenovirus is defective in cell killing and virus release of host cells (Tollefson et al., 1996). It is produced in large amounts during the last phase of viral infection by overcoming the cell death suppression function of E1B 19kDa. ADP (E3-11.6K) is Golgi apparatus and nuclear membrane localized. The exact mechanism of its cell killing activity is unknown.

#### 1.13.4.9 E4orf4 Protein

An adenovirus E4 region protein E4orf4 is a 14 kDa protein. E4orf4 can induce p53-independent apoptosis. Cell death induced by E4orf4 is essential for the efficient release of viral progeny from infected host cells. The ability of E4orf4 to induce apoptosis is linked to its interaction with the Bα subunit of PP2A (Lavoie et al., 1998; Marcellus et al., 1998). It binds to the Bα subunit of phosphatase 2A (PP2A) to regulate Ser/Thr phosphatase activity (Mumby and Walter, 1993). However, there is a possibility that E4orf4 is a substrate of the phosphatase PP2A and the activity of E4orf4 is regulated by its Ser/Thr phosphorylation status.

#### 1.13.4.10 E4orf6 Protein

Another E4 region encoded protein, E4orf6, is a 34 kDa nuclear protein, E4orf6 can bind to the p53/E1B 55K complex thus inhibiting p53 activity (Dobner et al., 1996). E4orf6 and E1B 55K work together to effectively inactivate and degrade p53.

## *1.14 Programmed Cell Death in Unicelluar Organism*

Recent research works illustrates that certain pathways of death in many non-metazoan organisms share morphological similarities with metazoan apoptosis. The developmentally regulated programmed cell death in Trypanosoma cruzi shows membrane 'blebbing',

chromatin condensation and margination to nucleus, and DNA cleavage into low-molecular-weight fragments. A Saccharomyces cerevisiae cdc 48 cell cycle regulator mutant strain also exhibits chromatin condensation and DNA fragmentation, as well as exposure of the internal membrane phospholipid phosphatidylserine (PtdSer) to the extracytoplasmic face of the plasma (Madeo et al., 1997). This raises the question of whether the dying yeast mutant has activated an endogenous cell death machinery which exhibits those apoptotic features of the metazoan. However, the whole genomic sequence of S.cerevisiae is now complete and the search for homologs of Ced-3, Ced-4, and Ced-9 was unsuccessful. Yeast also do not contain any other pro- or anti-apoptotic Bcl-2 family members. However, expression of Bax or Bak, two mammalian pro-apoptotic family members, in S.cerevisiae and S.pombe, can induce cytotoxicity and cell death (Greenhalf et al., 1996; Tao et al., 1997; Zha et al., 1996a). The killing effect is not due to non-specific toxicity because Bax or Bak mutants which cannot kill mammalian cells also fail to kill yeast cells, indicating that Bax/Bak cytotoxicity in yeast has something to do with their proapoptotic function. Although co-expression of Bcl-xL can inhibit Bax or Bak killing in both fission and budding yeast, a Bcl-xL mutant, which cannot interact with Bax, can still block the Bax killing in yeast, suggesting that Bax and Bcl-xL's antagonistic biological effects cannot be explained simply by their heterodimerization to each other (Tao et al., 1997). In a S.cerevisiae mutant strain which lacks oxidative phosphorylation, the killing effect of Bax has been severely diminished (Greenhalf et al., 1996). Another S.cerevisiae mutant which does not express $F_0F_1$-ATPase was also not killed by Bax expression (Matsuyama et al., 1998). Furthermore, Bax-induced yeast cell death is also associated with release of cytochrome c from the mitochondria (Manon et al., 1997). Together, this evidence indicates that the killing effect of Bax in yeast is reminiscent of its pro-apoptotic function in mammalian cells. Since no metazoan cell death machinery proteins such as Ced-3, Ced-4, or Ced-9 homologues have been found in yeast, it is possible that Bax and Bak are acting directly or indirectly on highly conserved unicellular cell death machinery components which target the same apoptotic counterparts of mammalian cells. Whether or not yeast contain a cell death machinery that is distinct from that in metazoan cells still needs to be determined. This lack of mammalian cell death machinery, however, makes yeast a powerful tool for complementary screening of novel mammalian regulators of cell

death machinery or for dissection and analysis of individual metazoan cell death proteins. A complementation screen for mammalian proteins suppressing Bax-induced killing of S.cerevisiae lead to the isolation of the BI-1 (Bax inhibitor protein-1) gene (Xu and Reed, 1998). BI-1 is an integral membrane protein which can also block apoptosis in mammalian cells (Xu and Reed, 1998).

## *1.15 Thesis Proposal*

The goal at the beginning of my Ph.D. studies were to better understand the mechanism of apoptosis and how the adenovirus E1B 19kDa functions in the apoptosis pathway.

At the beginning of these studies, very little was known about E1B 19kDa's function and mechanism in apoptosis. In Chapter 2, I showed that E1B 19kDa can function like Bcl-2 to block the cellular apoptosis induced by hygromycin B. I also presented data about the regulation of E1B 19kDa's anti-apoptotic function by other pro-apoptotic Bcl-2 family members such as Bax and Bad. These studies revealed that although E1B 19kDa is a structural and functional homolog of Bcl-2, it also has its own unique features.

During the course of my studies, an ER localized Bcl-2/Bcl-xL interacting protein, Bap31, was found. A series of E1B 19kDa chimeric proteins were made to study their ability to interact with Bap31 or Bcl-xL. This work revealed that the BH3 domain of E1B 19kDa has different protein-protein interaction properties than the Bcl-2 BH3 domain.

In the discussion, I will attempt to incorporate these data into a global picture about how E1B 19kDa may function in apoptosis regulation and additionally integrate it into our knowledge of apoptosis in mammalian cells.

# CHAPTER 2

## Induction of p53-independent apoptosis by hygromycin B: suppression by Bcl-2 and adenovirus E1B 19-kDa protein

EXPERIMENTAL CELL RESEARCH 221, 55–59 (1995)

# Induction of p53-Independent Apoptosis by Hygromycin B: Suppression by Bcl-2 and Adenovirus E1B 19-kDa Protein[1]

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**Hygromycin B, an aminoglycoside antibiotic that is widely used to establish stable mammalian cell lines that carry a bacterial gene conferring resistance to the drug, is shown here to induce apoptotic programmed cell death in susceptible cells. Dying cells exhibited typical features of apoptosis, including cell shrinkage, membrane blebbing, nuclear pyknosis, and extensive internucleosomal fragmentation of DNA. Employing concentrations of hygromycin B that are typically used for selecting stable cell lines, we show that susceptible cells die rapidly, exhibiting the morphological properties of apoptosis by 18 h and detectable DNA fragmentation as early as 2 h after receiving the drug. G418, on the other hand, required days to cause cell death, which was not accompanied by internucleosomal DNA fragmentation. Apoptotic cell killing by hygromycin B did not require expression of wild-type p53 and was suppressed by both Bcl-2 and the Adenovirus type 5 E1B 19-kDa protein.** 

## INTRODUCTION

Apoptotic cell death in multicellular organisms contributes to tissue homeostasis and remodeling during development. It also functions as a critical defense mechanism against cellular assaults, leading to the rapid removal of affected cells with little or no response from the organism as a whole [1–3]. This has proven to be particularly important where the assault involves autonomously replicating agents, like viruses, or uncontrolled clonal cell proliferation like that associated with cancer. It may be that most mammalian cells are poised to quickly engage an apoptotic pathway as part of a default mechanism and, therefore, require various suppressor signals to prevent its activation. Such suppressors can be exploited to successfully establish productive viral infections and oncogenesis in many instances [1–3].

In mammals, the best understood suppressors of apoptosis are those related to the proto-oncogene product of *bcl-2* [4–6]. High level expression of *bcl-2* occurs in most follicular B-cell lymphomas, where it contributes to neoplasia [7], and also in normal development where it is required for extended B-cell memory [8]. Ectopic expression of the gene has been found to inhibit apoptosis in response to a diverse array of natural and artificial signals [1,2]. Bcl-2 functions as a dominant suppressor of apoptosis, similar to that which has been defined genetically for the Bcl-2 homolog in *C. elegans*, the product of *ced-9* [9]. Not surprisingly, certain viruses encode suppressors of apoptosis as a means of escaping the host response [10]. The E1B region of Adenovirus, for example, encodes two gene products, the E1B 55-kDa protein and the E1B 19-kDa protein (19 K), both of which can inhibit apoptosis triggered by the E1A gene, although the 19-K protein does so much more effectively [11]. Bcl-2 can functionally substitute for E1B 19K in both viral infection [12, 13] and E1A-mediated cellular transformation [11].

Aminoglycoside antibiotics are well-established inhibitors of cell growth that function by inhibiting protein synthesis in both prokaryotes and eukaryotes [14]. Based on these properties, inhibitors like hygromycin B and G418 at high concentrations have been widely used in selection strategies for expression of transfected genes in eukaryotic cells, due to the introduction in the expression vectors of bacterial genes that confer resistance to the antibiotic [15, 16]. Here, we demonstrate that hygromycin B is a potent inducer of apoptosis and that the apoptotic response to the drug does not require a functional p53 protein. Both Bcl-2 and adenovirus E1B 19K confer resistance to the cytotoxic effects of hygromycin B, at least over the time course in which the normal cell dies in response to this treatment.

## MATERIALS AND METHODS

*Reagents.* Hygromycin B was purchased from Calbiochem (La Jolla, CA), G418 (geneticin) from Gibco-BRL (New York, NY), and actinomycin D and cycloheximide were from Sigma (St. Louis, MO).

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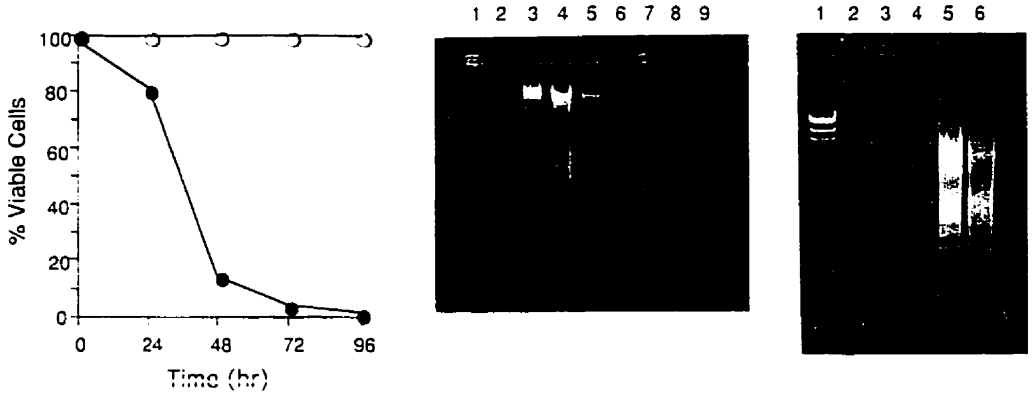


FIG. 1. Effects of hygromycin B and G418 on CHO LR73 cells. (Left) Cells were grown to 80% confluency and subsequently incubated either with (●) or without (○) 800 μg/ml hygromycin B. At the times indicated, cell viability was assayed by determining the percentage of cells that excluded trypan blue. (Middle) Cells were maintained for 18 h in the absence (lane 1) or presence of various concentrations of hygromycin B (20 μg/ml, lane 2; 80 μg/ml, lane 3; 400 μg/ml, lane 4; 1 mg/ml, lane 5) or G418 (10 μg/ml, lane 6; 100 μg/ml, lane 7; 500 μg/ml, lane 8; 2 mg/ml, lane 9). Low-molecular-weight DNA was isolated and analyzed by agarose gel electrophoresis. (Right) Cells were either untreated (lane 2) or incubated with 800 μg/ml hygromycin B for 2 h (lane 3), 8 h (lane 4), 11 h (lane 5), or 24 h (lane 6). Low-molecular-weight DNA was isolated and analyzed by agarose gel electrophoresis. Lane 1, Lambda DNA digested with *Hin*dIII.

*Cell culture and transfection.* CHO LR73 cells were grown in αMEM containing 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Treatments were initiated when cultures reached approximately 80% confluency. cDNAs encoding human Bcl-2 [12] or Adenovirus type 5 E1B 19-kDa protein [34] were introduced into the Rc/RSV eukaryotic expression vector (Pharmacia), which also encodes the neomycin resistance gene. The cDNAs were modified to encode methionine hexahistidine at the $NH_2$-terminus of Bcl-2 and influenza hemagglutinin (HA) epitope, MAYPDYVPDYAV, at the COOH-terminus of E1B 19K. The sequences of all constructs were verified by DNA sequencing. Following transfection by the $CaCl_2$ procedure [17], stable CHO LR73 lines expressing either protein were selected in G418 and screened for Bcl-2 and E1B 19K expression employing monospecific antisera.

*Cell viability.* A total of $10^5$ cells in 1 ml culture medium were plated per well in multi-well dishes and grown to approximately 80% confluency. Fresh medium ± hygromycin B or G418 was added. At various times thereafter, the supernatants were removed, the attached cells obtained following trypsinization and combined with the supernatants, and total cells recovered by centrifugation. Pellets were suspended and assayed under a microscope for percentage of cells that excluded trypan blue.

*DNA fragmentation.* Cells were collected following trypsinization and low-molecular-weight DNA obtained by the method of Hirt [18]. Following electrophoresis in a 1.8% agarose gel, the DNA was stained with ethidium bromide, exposed to uv light, and photographed.

*Electron microscopy.* Cells were fixed *in situ* with 2% (v/v) glutaraldehyde, postfixed in 1% (w/v) osmium tetroxide, 1% (w/v) potassium ferrocyanide, and 0.1 *M* Na cacodylate, pH 7.4, stained *en bloc* with 1% (w/v) uranyl acetate, and embedded in epon. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Phillips 300 electron microscope.

## RESULTS AND DISCUSSION

### *Hygromycin B Induces Apoptosis in CHO LR73 Cells*

Cell viability following treatments with or without drug was determined by their ability to exclude trypan blue. As shown in Fig. 1 (left), CHO LR73 cells rapidly succumbed to 800 μg/ml hygromycin B, with greater than 95% of the cells routinely observed to die within 48 h. The concentration of hygromycin that was employed is similar to that used for selection of stable cell lines expressing resistance to the drug.

To determine if the observed cell death correlated with apoptosis, both DNA content and cellular morphology were examined. Treatment of cells over a wide concentration of hygromycin B (20 μg–1 mg/ml) resulted in the appearance of an oligonucleosomal ladder upon analysis of DNA by gel electrophoresis (Fig. 1, middle, lanes 1–5), a response that has been widely associated with apoptosis [19, 20]. At 800 μg/ml hygromycin B, significant DNA fragmentation occurred by 2 h, with maximal appearance of an oligonucleosomal ladder occurring at 11 h after receiving the drug (Fig. 1, right). DNA fragmentation, therefore, occurred at a relatively early stage, at a time when the majority of cells receiving the drug contained an intact cell membrane (Fig. 1, left). By 18 h, however, the cells exhibited morphological features typical of apoptosis [21]. This included cell shrinkage, extensive membrane blebbing, and nuclear pyknosis (Fig. 2).

In contrast to hygromycin B, cells that received the drug G418 took longer to lose viability, requiring 6–7 days for 50% of the cells to die (not shown). As expected, 18 h after receiving this drug, CHO LR73 cells exhibited negligible, if any, fragmentation of DNA (Fig. 1, middle, lanes 6–9). Moreover, DNA fragmentation was not observed even after cells had been incubated for 7 days with G418.

### *Bcl-2 and Adenovirus E1B 19-kDa Protein Prevent Apoptosis Caused by Hygromycin B*

Stable CHO LR73 cell lines were established that express either human Bcl-2 or Adenovirus type 5 E1B

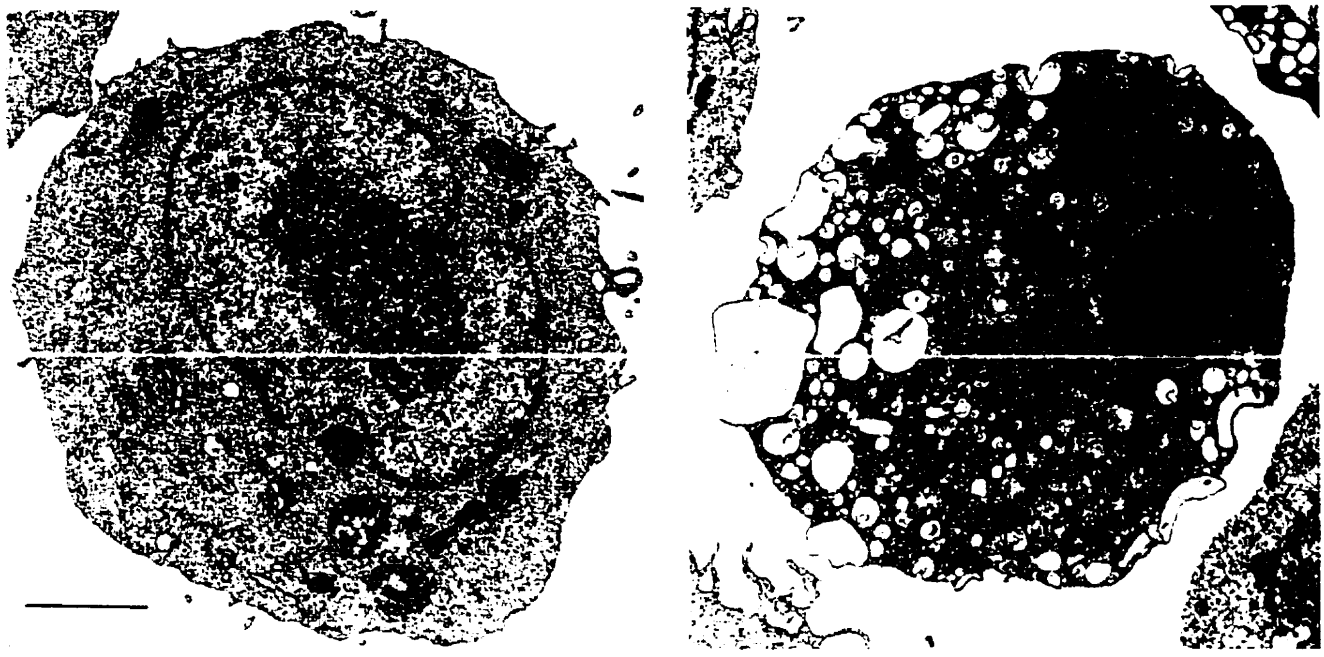

FIG. 2. Electron micrographs of CHO LR73 cells that were untreated (left) or treated for 18 h with 400 μg/ml hygromycin B (right). Bar, 1.54 μm.

19-kDa protein, employing expression vectors that confer neomycin (G418) resistance for selection (Fig. 3). Like nonselected cells (Fig. 1), CHO LR73 cells carrying just the neomycin resistance gene were rapidly killed by hygromycin B (Fig. 4, left), resulting in apoptotic DNA fragmentation (right, lane 2). Both Bcl-2 and E1B 19K conferred protection against the drug and prevented the appearance of oligonucleosomal fragments (Fig. 4). A number of independently derived cell lines expressing Bcl-2 or 19K were obtained and found to exhibit this property. The extent of protection against Hygromycin B, however, correlated with the level of Bcl-2 or 19K expressed in the individual cell lines.

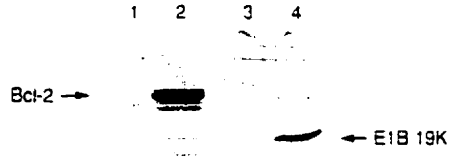


FIG. 3. CHO LR73 cell lines that express Bcl-2 or E1B 19-kDa protein. Stable cell lines were created following transfection with plasmids that encode a neomycin resistance gene either alone (lanes 1 and 3) or together with cDNAs that encode either human Bcl-2 (lane 2) or Adenovirus type 5 E1B 19-kDa protein (19K) tagged with the HA epitope (lane 4), as described under Materials and Methods. Proteins from whole cell extracts were separated by SDS–PAGE, transferred to nitrocellulose, and the blots incubated either with 6C8 monoclonal antibody against Bcl-2 [12] (lanes 1 and 2) or with 12CA5 monoclonal antibody against the HA epitope [12] (lanes 3 and 4). Following incubation with secondary antibodies conjugated to alkaline phosphatase, color was developed with NBT/BCIP (Boehringer-Mannheim) according to the manufacturer's instructions. The positions of Bcl-2 and E1B 19K are indicated.

### *Induction of Apoptosis by Hygromycin B Occurs Independently of p53*

p53A is a cell line derived from baby rat kidney cells that expresses a temperature-sensitive mutant form of p53 [22]. At the permissive temperature, wild-type p53 is expressed and the cells spontaneously die [22]. At the nonpermissive temperature (38°C), only the mutant form of p53 is expressed, the cells are viable and are resistant to agents causing p53-dependent apoptosis [22]. When treated with hygromycin B, however, these cells died at the nonpermissive temperature (38°C) within 47–72 hours, but remained completely viable in the absence of drug (not shown). As depicted in Fig. 5, sensitivity to hygromycin was associated with apoptotic DNA fragmentation. Induction of apoptosis by hygromycin B, therefore, does not require p53.

## CONCLUSIONS

Hygromycin B has been widely used in mammalian cell cultures for selection of transfected genes that are coexpressed with a hygromycin resistance gene. Little is known, however, about the mechanism by which sen-

sitive cells die in response to the drug. Here, we have shown that CHO LR73 cells rapidly lose viability following treatment with hygromycin B, by a process that is accompanied by oligonucleosomal fragmentation of DNA, nuclear pyknosis, and extensive membrane blebbing, all of which are hallmark features of apoptosis. This response to hygromycin B does not depend on a functional p53 gene product. Although p53-induced growth arrest and apoptosis are well-known cellular responses to DNA damage [23–25], it is also known that apoptotic pathways can be induced which are independent of p53, both during normal developmental programs [26, 27] and in response to a number of genotoxic agents [28]. Hygromycin B belongs to this p53-independent category. Paradoxically, however, both cycloheximide and actionomycin D, inhibitors of protein and RNA synthesis, respectively, blocked the apoptotic response to hygromycin (not shown), despite the fact that hygromycin B itself is an inhibitor of protein synthesis. It is not clear, therefore, if the mechanism of induction of apoptosis by hygromycin relates to its effect on protein synthesis or on some other pathway. Finally, hygromycin-induced apoptosis is blocked by Bcl-2, a suppressor of apoptosis which is known to function far downstream on an apoptotic death pathway that can be triggered by a diverse array of external and internal signals [29, 30]. E1B 19K, on the other hand, is a viral suppressor of apoptosis which has evolved specifically to counter the cytotoxic effects of Adenovirus E1A gene expression, thus allowing E1A-induced host DNA synthesis to proceed [31]. Although Bcl-2 has been shown to substitute for E1B 19K in E1A-mediated events [31],

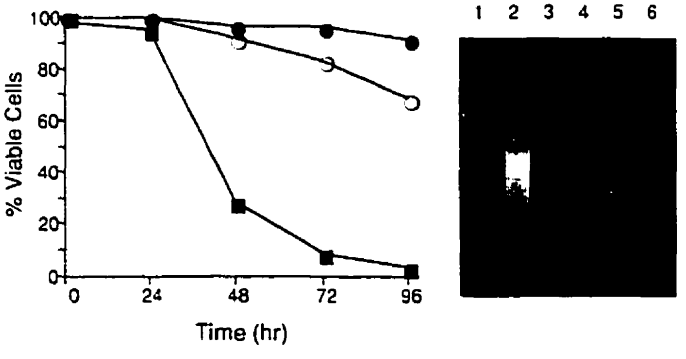


**FIG. 4.** Suppression of hygromycin-induced apoptosis by Bcl-2 and Adenovirus E1B 19-kDa protein. Stable CHO LR73 cell lines were established that express the bacterial *neomycin* resistance gene either alone (■) or with the human bcl-2 (●) or Adenovirus E1B 19K (○) gene. (Left) Cells were incubated for the indicated times in the presence of 400 μg/ml hygromycin B, and cell viability assayed by exclusion of trypan blue. (Right) Cells expressing the *neomycin* resistance gene either alone (lanes 1 and 2) or with E1B 19K (lanes 3 and 4) or bcl-2 (lanes 5 and 6) gene were maintained for 18 h in the absence (lanes 1, 3, and 5) or presence of 800 μg/ml hygromycin B (lanes 2, 4, and 6). Low-molecular-weight DNA was isolated and analyzed by agarose gel electrophoresis.


**FIG. 5.** Induction of apoptosis by hygromycin B occurs independently of p53 expression. The p53A cell line [22] was maintained at the nonpermissive temperature (38°C) and incubated for 18 h in the absence (lane 2) or presence of 400 μg/ml hygromycin B (lane 3). Low-molecular-weight DNA was isolated and analyzed by agarose gel electrophoresis. Lane 1, Lambda DNA digested with *Hin*dIII.

and in a limited number of other circumstances [32, 33], the extent of the similarity in the ability of Bcl-2 and E1B 19K to suppress apoptosis has not been fully examined. The fact that E1B 19K also counters hygromycin-induced cell death, however, suggests that, like Bcl-2, it too may confer protection against apoptosis in diverse circumstances.

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# CHAPTER 3

## Adenovirus E1B 19-kDa death suppressor protein interacts with Bax but not with Bad

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# Adenovirus E1B 19-kDa Death Suppressor Protein Interacts with Bax but Not with Bad*

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**Adenovirus E1B 19-kDa protein (19K) is a member of the Bcl-2 family of suppressors of apoptosis. The suppressors function through heterodimerization with the death promoters, Bax and related proteins, thus establishing a set point within the cell that determines whether or not apoptosis is executed in response to a death signal. Sequence similarities between 19K and Bcl-2 are largely restricted to short Bcl-2 homology (BH) domains that mediate interaction with Bax. The BH1 sequence in 19K is degenerate but nevertheless contains a conserved glycine residue found in all family members that when mutated to alanine in Bcl-2 results in loss of Bcl-2 function and ability to dimerize with Bax (Yin, X.-M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) *Nature* 369, 321–323). Here, we show that the analogous mutation in BH1 of 19K also abrogates the anti-apoptotic properties of 19K and its ability to interact with Bax, thus establishing the critical importance of this residue within BH1 and the likely similarity of Bcl-2 and 19K function. In distinct contrast to Bcl-2, however, 19K interaction was not detected with Bad, a Bcl-2/Bcl-$X_L$ dimerizing protein that can potentially regulate a Bax·Bcl-2/Bcl-$X_L$ survival set point and reinstate susceptibility to a death signal. Furthermore, the anti-apoptotic function of 19K was not overcome by enforced expression of Bad in transfected cells. This feature of 19K may provide adenovirus with a selective advantage in evading premature induction of apoptosis by the host cell.**

Apoptotic cell death is essential for tissue modeling and homeostasis in metazoans and provides the organism with the ability to selectively remove cells that are targets of various types of growth deregulating events (1, 2). Examples of such events include activation of oncogene expression and viral infection (3). Although the demise of the cell is initially triggered upon receipt of a specific death signal, execution of the apoptotic pathway occurs only upon activation of critical Ced-3/ICE cysteine proteases, which cleave and inactivate/activate specific target molecules (4–6). Whether or not the Ced-3/ICE pathway is activated by a death signal, however, depends on the status of various cellular regulators of apoptosis (1–3).

The prototypic and best studied examples of regulators of apoptosis are those associated with the Bcl-2/Bcl-$X_L$ (7–9) and Bax/Bak (10–13) family of suppressors and promoters, respectively. Bax was first discovered as a member and heterodimerizing partner of Bcl-2 (10). Together, the heterodimerizing partners establish a set point within the cell that determines whether or not the cell will ultimately respond to a death signal. Homo- and heterodimerization is dependent upon short Bcl-2 homology (BH)[1] domains within the polypeptides, one of which (BH1) is critical for Bax·Bcl-2 heterodimerization but not Bcl-2 homodimerization (14). Bad, a more distantly related protein that contains BH domains, is a direct regulator of the set point (15). It heterodimerizes with Bcl-2/Bcl-$X_L$ but does not interact with itself or with Bax. Thus, it has the potential to readjust a Bax·Bcl-2/Bcl-$X_L$ set point that would otherwise specify cell survival following a death signal to one that specifies apoptosis (15). Although it is not presently known how the set point controls whether or not the cell will engage the apoptotic program, recent studies have shown that Bcl-2/Bcl-$X_L$ operates either at or upstream of processing of pro-CPP32/apopain/Yama (16, 17), a Ced-3/ICE protease that may be responsible for initiating the cascade of events that ultimately leads to the final execution of apoptosis (18–20). In this regard, the position of the set point within the apoptotic pathway differs from that of the viral apoptotic inhibitors, cowpox CrmA (4, 5) and Bacculovirus p35 (21), which function as direct active site inhibitors of various Ced-3/ICE proteases. However, it is similar to that of E1B 19-kDa protein (16), an adenovirus suppressor of apoptosis that is functionally interchangeable with Bcl-2 in several different contexts (22–25).

Adenovirus has provided an important model system for studies of the relationship between cellular transformation and apoptosis, because the viral gene that induces host cell DNA synthesis and immortalization, E1A, is also a potent inducer of apoptosis (22). To permit the transforming properties of E1A to be manifested, adenovirus encodes two negative regulators of apoptosis: E1B 55-kDa protein, which binds p53 and impairs its ability to regulate gene expression (26, 36) and mediate E1A-induce apoptosis (27, 28), and E1B 19-kDa protein, a general suppressor of both p53-dependent and -independent cell death (27–30). Despite the functional similarities between Bcl-2 and E1B 19K, the two proteins are quite different over most of their respective polypeptide sequences, with the exception of regions that are related to BH1 and BH3 (31, 32). These regions in 19K, however, mediate interaction with Bax (31, 32), suggesting that Bax·19K establishes an apoptotic control set point that is analogous to that of Bax·Bcl-2/Bcl-$X_L$. Consistent

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[1] The abbreviations used are: BH, Bcl-2 homology; 19K, 19-kDa protein encoded by adenovirus serotype 5 early region 1B; HA, influenza virus hemagglutinin epitope; PAGE, polyacrylamide gel electrophoresis.

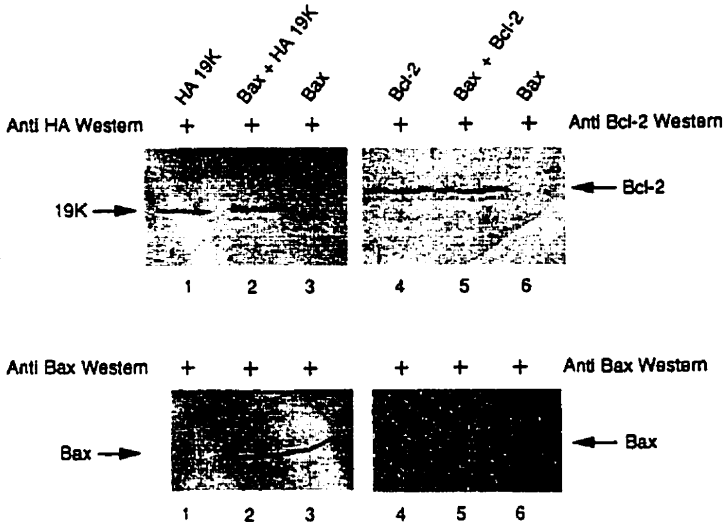


FIG. 1. **Co-expression of *BAX* and either *19K* or *BCL-2*.** Stable CHO LR73 cell lines were created that contain HA 19K, Bcl-2, or Bax or both Bax and either HA 19K or Bcl-2 (see "Materials and Methods"). Total cell extracts containing equivalent levels of protein were subjected to SDS-PAGE, transferrred to nitrocellulose, and incubated (*plus sign*) with 12CA5 anti-HA (*upper left, lanes 1–3*), 6C8 anti-Bcl-2 (*upper right, lanes 4–6*), or P-9 anti-Bax (*lower panels, lanes 1–6*). Blots were visualized following incubation with secondary antibodies conjugated to alkaline phosphatase and treatment with NBT/BCIP. The positions of 19K, Bcl-2, and Bax are indicated.

with this thesis, enforced expression of either Bax or Bak overcomes the death protective effect provided by 19K (12, 31, 32). In the case of Bcl-2, a relatively conservative mutation within the BH1 domain, which changes glycine at position 145 to alanine, abrogates the death suppressor function and ability of Bcl-2 to interact with Bax (14). Although the corresponding 19K BH1 domain is relatively degenerate compared with other family members, it contains the analogous glycine. We show here that mutation of this glycine to alanine has the same detrimental effect on 19K, which implicates this highly conserved amino acid position within all known BH1 domains as critically important for function. Of note, however, we also find that in distinct contrast to Bcl-2 and Bcl-$X_L$, interaction of 19K with Bad was not detected. This has particular significance because it suggests that the polypeptide sequence of 19K has evolved to avoid negative regulation by Bad, a feature that may provide adenovirus with a selective advantage in evading premature killing of the host cell.

## MATERIALS AND METHODS

*Cells and Expression Vectors*—Standard recombinant polymerase chain reaction methodology was employed to insert oligonucleotides encoding the HA epitope, (M)AYPDYVPDYAV, at the 3'-end and 5'-end, respectively, of the protein-coding sequence of *E1B 19K* and *BCL-2* cDNA (25, 30). cDNAs encoding human Bcl-2 (7) or HA Bcl-2 (25), adenovirus serotype 5 HA 19K (30), mouse Bax (10), or mouse Bad (15) were cloned into the Rc/RSV vector (Pharmacia Biotech Inc.) carrying a neomycin resistance gene or into pLXSH vector carrying a hygromycin resistance gene. The authenticity of all constructs was verified by DNA sequencing. The vectors were transfected into CHO LR73 cells, as described previously (30), and stable transfectants obtained by selection in G418 or hygromycin B and identified by Western blot analysis. Cell lines expressing various levels of the individual proteins were isolated. To establish co-expressing lines, cellular clones that had been selected in one drug were subsequently transfected and selected in the other drug and characterized by Western blotting.

*Cell Viability Assays*—Cells were grown to ~80% confluency in α-minimum Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and streptomycin. Puromycin (50 μg/ml) or staurosporin (6.0 μM) were added, total cells subsequently were collected at various times, and the percentage of the cells that excluded trypan blue was determined in two independent experiments, and the results were averaged as described (25, 30). All experiments were conducted 3–5 times and yielded results very similar to those presented.

*Western Blotting*—Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with P-9 rabbit polyclonal antibody against Bax, 12CA5 mouse monoclonal anti-HA, 6C8 hamster monoclonal anti-Bcl-2, rabbit polyclonal anti-19K, or rabbit polyclonal sc-943 anti-Bad. Following incubation with secondary antibody conjugated to alkaline phosphatase, color was developed with NBT/BCIP (Boehringer Mannheim) according to the manufacturer's instructions.

*Co-immunoprecipitation*—Cell lines containing equivalent amounts of HA-Bcl-2, HA-19K, or neither protein (*neo* controls) were grown to ~80% confluency, collected following trypsinization, and washed, and the cells were homogenized at 4 °C in medium containing 2% (w/v) Nonidet P-40, 140 mM KCl, 5 mM $MgCl_2$, 1 mM ethylene glycol-*bis*-(β-aminoethylether)N,N'-tetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 10 mM Hepes, pH 7.2. Following centrifugation at 12,000 × *g* for 15 min, supernatants were mixed with an equal volume of reticulocyte lysate containing [$^{35}$S]Bax or [$^{35}$S]Bad transcription-translation product (34), gradually diluted with 4.5 volumes of homogenization medium lacking detergent, and incubated for 1 h at 4 °C prior to addition of 12CA5 anti-HA. Immune complexes were recovered with protein A-Sepharose and analyzed by SDS-PAGE and fluorography.

## RESULTS AND DISCUSSION

Because Bcl-2 functions as a heterodimerizing partner with Bax (10), the ratio of the two proteins, rather than absolute levels, is an important determinant of the final outcome for a cell that receives a death signal (1, 10). This is also true for other Bax-interacting members of the Bcl-2 family (15, 31, 32). To study the ability of enforced expression of Bax to overcome the activity of a death suppressor, therefore, it is important to compare these co-expressing cells with ones that express the same level of the death suppressor in the absence of Bax. To that end, stable CHO LR73 cell lines were established that express equivalent levels of 19K plus or minus Bax (Fig. 1, *upper panel, lanes 1* and *2*) or equivalent levels of Bcl-2 plus or minus Bax (Fig. 1, *upper panel, lanes 4* and *5*), as judged by Western blot analysis. Cell lines were also generated that contain Bax alone, at levels that are equivalent to the level of Bax in the co-expressing cell lines (Fig. 1, *lower panel*, compare *lane 2* with *lane 3* and *lane 5* with *lane 6*). A number of such cell lines were created and each gave relative responses to death signals that were comparable with those described below.

*Elevation of Bax Overcomes the Death Suppressor Effects of Both Bcl-2 and E1B 19K*—The stable cell lines that were analyzed in Fig. 1 by Western blotting were also examined for their susceptibility to a potent inducer of apoptotic cell death, puromycin (35). In common with other systems (10), enforced expression of *BAX* alone had only a slight enhancing effect on the rate of cell death in response to the drug relative to *neo* control cells but overcame the otherwise protective effect against the drug that was conferred by Bcl-2 (Fig. 2, *upper graph*). Whereas cells expressing *neo*, either alone or together with *BAX*, were

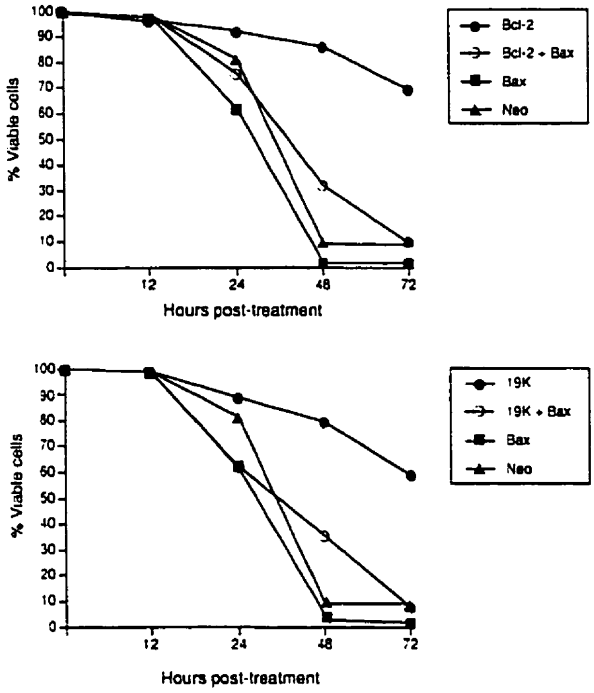


FIG. 2. **Enforced expression of *BAX* overcomes the anti-apoptotic activity of Bcl-2 and E1B 19K.** CHO LR73 cell lines were generated that express *neo* either alone (▲) or together with *BAX* (■), or *BCL-2* or *19K* alone (●) or both *BAX* and either *19K* or *BCL-2* (□). The cells were treated with 50 μg/ml puromycin, and at the indicated times following the addition of drug, cells were collected, and the percentage of viability determined by exclusion of Trypan Blue.

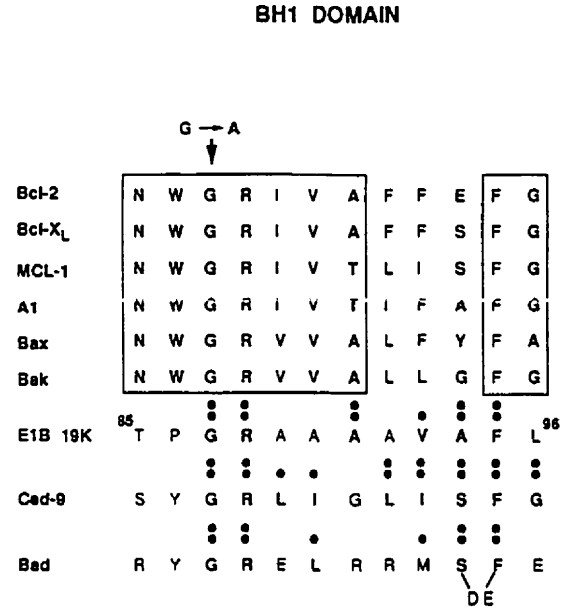


FIG. 3. **E1B 19K contains a degenerate BH1 domain.** Amino acid sequences (single-letter code) of the BH1 domains of various members of the Bcl-2 family (from Ref. 1) are shown and compared with amino acids 85–96 of 19K of adenovirus serotype 5. *Boxes* denote highly conserved regions. *Single* and *double dots* denote amino acids in 19K, Ced-9, and Bad that are either similar or identical, respectively, to amino acids in the BH1 domains of the other proteins. *Arrow* denotes a glycine to alanine mutation (see text).

almost all killed by 48 h after receiving 50 μg/ml of the drug, approximately 85% of the cells that express *BCL-2* remained viable during this period. Co-expression of *BAX* with *BCL-2*, however, reduced the cell survival level to 30–35% at 48 h after receiving puromycin, and this was reduced to the level of *neo* controls by 72 h. A very similar pattern and extent of protection against puromycin and abrogation of this protection by Bax were also observed for E1B 19K (Fig. 2, *lower panel*).

*A Degenerate BH1 Domain Contributes to the Anti-apoptotic Function of E1B 19K and Ability to Interact with Bax*—Fig. 3 shows the sequences of BH1 domains in various Bcl-2 family members (from Ref. 1) and aligns these regions to a sequence in adenovirus serotype 5 E1B 19K (37) from amino acid 85 to 96 that shows similarity to the BH1 domains in these other proteins. The similarity, however, is relatively weak and in fact is less than that observed for the evolutionarily distant Bcl-2 homolog in *Caenorhabditis elegans*, Ced-9 (14, 33) (Fig. 3). Nevertheless, this predicted BH1 domain of 19K contains the highly conserved GR, which is present in all family members and whose glycine moiety is important for function. A relatively conservative change of this glycine to alanine at residue 145 of Bcl-2 results in loss of both Bcl-2 function and its ability to heterodimerize with Bax (14). Therefore, to determine if the predicted BH1 domain of 19K is of functional significance, the same mutation was introduced at residue 87 of 19K (designated 19K G87A) (Fig. 3). Several cell lines were created in CHO LR73 that express various levels of the mutant 19K protein, and in all cases, the G87A mutation was found to result in loss of 19K protection against puromycin treatment compared with an equivalent level of wild-type 19K. An example of such an analysis is shown in Fig. 4*A*, in which the level of HA 19K G87A was similar to or even greater than wild-type HA 19K as judged by immunoblotting with anti-HA antibody (Fig. 4*A*, *inset*). The 19K wild-type and G87A cell lines described in Fig. 4 were also examined for their susceptibility to staurosporin, a protein kinase inhibitor that induces apoptosis in many cell types (38, 39). Again, 19K G87A exhibited a significant loss in the ability to prevent cell death compared with wild-type 19K (Fig. 5). Consistent with the loss of death suppressing activity resulting from the G87A mutation of 19K, the mutant 19K in these cells exhibited a corresponding reduction in its ability to interact with Bax, relative to equivalent levels of wild-type 19K, in a co-immunoprecipitation assay *in vitro* (Fig. 4*B*, compare *lanes 3* and *4*).

*Bcl-2, but Not E1B 19K, Interacts with Bad*—Bad competes with Bax for heterodimerization with Bcl-2 or Bcl-$X_L$ and therefore has the potential to adjust a survival control set point to one that permits apoptosis to occur (15). In co-expressing cells, Bad overcomes the protective effects of Bcl-$X_L$ (15). However, despite repeated attempts, we have found that enforced expression of Bad does not overcome the death suppressor activity of E1B 19K (not shown). In distinct contrast to Bcl-$X_L$ and Bcl-2, interaction between 19K and Bad was not observed in a co-immunoprecipitation assay that readily detected interaction between Bcl-2 and Bad (Fig. 6, *right panel*, compare *lanes 3* and *4*) and interaction between 19K and Bax (Fig. 4*B*). In Fig. 6, cell extracts were employed that contained equivalent levels of HA Bcl-2 and HA 19K, as judged by Western blot analysis with 12CA5 anti-HA (Fig. 6, *left panel*). Similar results were obtained using different cell lines and cell types (CHO LR73 and human KB). The findings strongly suggest, therefore, that E1B 19K is not susceptible to regulation by Bad.

*Conclusions*—The early events of adenovirus infection require that the cytotoxic consequences of E1A expression are countered by negative regulators of apoptosis. To ensure this outcome, the virus encodes its own general suppressor of apoptosis, E1B 19K. The major role of 19K during viral infec-

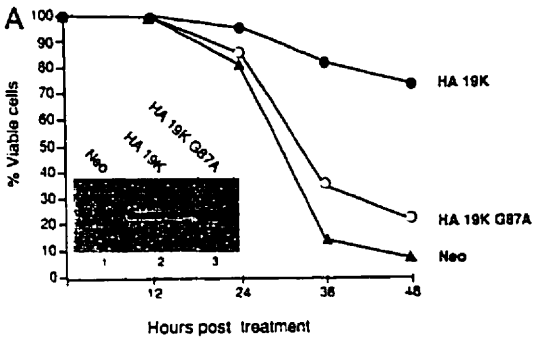


FIG. 4. **Mutation of glycine 87 inhibits E1B 19K function and ability to interact with Bax.** *A*, standard polymerase chain reaction methodology was employed to convert codon 87 in the protein coding sequence of Rc/RSV HA 19K from glycine (GGG) to alanine (GCG). Stable CHO LR73 cell lines were then obtained that express *neo* either alone (▲) or with wild type (●) or mutant G87A (○) *HA 19K* and examined for cell viability after treatment with puromycin as described in Fig. 2. *Inset*, total cell extracts from the *neo* alone (*lane 1*), *HA 19K* (*lane 2*), and *HA 19K G87A* (*lane 3*) lines containing equivalent amounts of protein were analyzed by Western blotting with anti-HA antibody (see "Materials and Methods"). *B*, [$^{35}$S]Bax was produced by transcription-translation (*lane 1*) in a rabbit reticulocyte lysate, and equal volumes were added to extracts from the cell lines assayed in *A*. Extracts were prepared in 2% Nonidet P-40 and contained equivalent amounts of HA 19K (*lane 3*) or HA 19K G87A (*lane 4*), or they contained neither protein (*Control*, *lane 2*). Following dilution of extracts to a final concentration of 0.1% detergent, 12CA5 anti-HA was added. Immmunocomplexes were collected, resolved by SDS-PAGE, and analyzed by autoradiography. The position of [$^{35}$S]Bax is indicated (*arrow*).

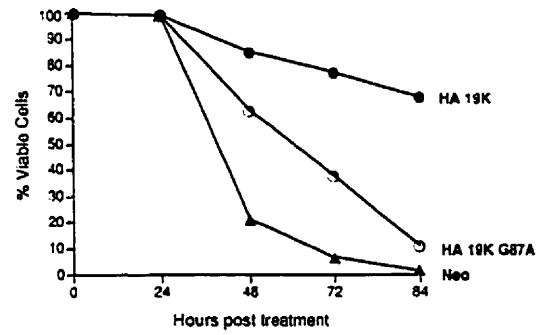


FIG. 5. **Mutation of glycine 87 inhibits the ability of E1B 19K to counter staurosporin-induced cell death.** The cell lines analyzed in Fig. 4*A* were also examined following treatment with 6.0 μM staurosporin, as indicated. Methodology and symbols are as described in the legend to Fig. 4*A*.

tion is to block p53-dependent apoptosis induced by E1A (22, 27–29) and p53-independent cell death that would otherwise be induced by an E4 viral gene product that is regulated by E1A (29). Based on evidence presented here and elsewhere (31, 32), 19K is both a structural and functional homolog of Bcl-2. Moreover, like Bcl-2, 19K appears to function within a signaling

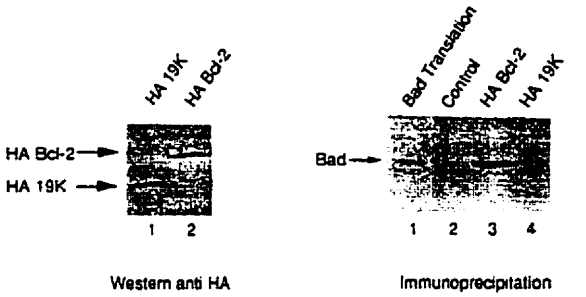


FIG. 6. **Bad interacts with Bcl-2 but not with E1B 19K.** *Right*, co-immunoprecipitation assays were conducted as described in Fig. 4*B*, except that [$^{35}$S]Bad (*lane 1*, 2.5% of input) was added to cell extracts containing equivalent amounts of HA Bcl-2 (*lane 3*), or HA 19K (*lane 4*), or containing neither protein (*Control*, *lane 2*). The position of [$^{35}$S]Bad is indicated (*arrow*). *Left*, Western blot of total cell extracts from *HA 19K* (*lane 1*) and *HA Bcl-2* (*lane 2*) cells containing equivalent amounts of protein and visualized with anti-HA antibody (see "Materials and Methods").

pathway that prevents processing and activation of pro-CPP32 in response to a death stimulus (16). Because similarities between the two proteins are restricted to limited regions within the molecules, including BH1 and BH3 (31, 32), comparisons between the two proteins provide an opportunity to correlate function with domain structure. Regions of sequence conservation and divergence within BH1 of Bcl-2 and 19K, for example, coupled with the deleterious mutation of the common glycine within this motif, help to define the essential features of this domain. Of particular relevance, however, we did not detect interaction between 19K and Bad, under conditions where 19K-Bax and Bcl-2-Bad interactions are readily observed. This has obvious evolutionary implications for the ability of the virus to evade potential host defense mechanisms, because it permits 19K to target Bax while at the same time avoiding disruption of this interaction and re-instatement of the apoptotic program by Bad.

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# CHAPTER 4

## Swapping the BH3 domain of Adenovirus E1B 19kDa protein with that of Bcl-2 converts 19K into a Bap31 and Bcl-xL associating protein

## *4.1 Abstract*

The Bcl-2 Homology domains (BH1, BH2, BH3, BH4) of Bcl-2 family proteins play important roles in the regulation of protein:protein interactions and apoptosis. Adenovirus E1B 19kDa protein (19K), a functional homologue of Bcl-2, has been reported to share weak sequence homology with other Bcl-2 family members at the BH1 and BH3 regions. Our sequence alignment analysis showed that 19K also contains a BH4 region overlapping its BH3 domain. Bap31, a 28kDa polytoptic integral protein of the endoplasmic reticulum (ER), is part of an ER protein complex that also includes Bcl-2/Bcl-xL, procaspase-8, and a CED-4-like protein ((Ng et al., 1997; Ng and Shore, 1998). E1B 19kDa does not interact with Bap31; nor does it interact with Bcl-2/Bcl-xL. We have made a set of chimeric E1B 19kDa proteins carrying different BH domains from Bcl-2 to test their protein-protein interaction properties. We show that the BH3 domain of Bcl-2, when substituted for the homologous region of 19K, confers the properties of interaction with Bap31 and Bcl-xL onto 19K. The subcellular location of the E1B 19kDa / Bcl-2 BH3 chimeric was very similar to that of wild type 19K. This chimeric protein retained its anti-apoptotic function. These results show that the BH3 domain of Bcl-2/Bcl-xL has an important role in mediating Bcl-2/Bcl-xL and Bap31 interactions. It also demonstrates that the BH3 domain of Bcl-2 controls different protein-protein interaction profiles compared to the BH3 domain of E1B 19kDa.

## 4.2 *Introduction*

Apoptosis, or programmed cell death, is a controlled cell death process that controls normal developmental processes, the maintenance of tissue homeostasis, and important host defense mechanisms. In the nematode *C.elegans*, several genes have been isolated with regulation effects on the general death program. Among them, Ced-9 inhibits apoptosis; and Ced-3, and Ced-4 promote cell death (Hengartner et al., 1992; Yuan and Horvitz, 1992; Yuan et al., 1993). Of note, all three C.elegan genes, Ced-3, Ced-4, and Ced-9, have mammalian counterparts. Ced-3 belongs to a family of proteases now named caspases that exist in mammals (Yuan et al., 1993). The caspase family consists of more than 13 members and the list is still growing. Caspases play fundamental roles in the execution of cell death. The proteolytic activation of initiator pro-caspases, such as procaspase-8 and –9 which have a long prodomain at their N-terminal, in the death triggered cell can subsequently lead to cleavage and activation of the downstream effector caspases, such as caspase-3 and –7, which carry short prodomains at their N-termini, to further amplify the caspase activation cascade (Cryns and Yuan, 1998). Effector caspases can then cleave their cellular target proteins such as CAD/CAPAN, nuclear lamin, PARP, Rb, and numerous other proteins , leading to cell death by ' thousands of cuts' (Cryns and Yuan, 1998). Ced-9 is a member of the mammalian Bcl-2 family of suppressor proteins that contains more than 15 members (Hengartner and Horvitz, 1994). Apaf-1 is a Ced-4 homologue identified in mammals (Zou et al., 1997). Like Ced-4, Apaf-1, is an adapter protein that mediates interactions between Bcl-2 family members and a caspase, procaspase-9. The current model for Ced-3 activation is that Ced-4 can bind to Ced-3 and induce the autoproteolytic activation of Ced-3 in *C.elegans* (Yang et al., 1998). Ced-3, Ced-4, and Ced-9 form a ternary protein complex. The binding of Ced-9 to Ced-4 can prevent the autocatalytic activation of Ced-3 by preventing the oligomerization of Ced-4 molecules associated with Ced-3 (Yang et al., 1998).

Two complexes controlling caspase activation in mammals have been identified. In one, cell death receptors, such as Fas/APO-1/CD95 (Smith et al., 1994), TNFR1 (Smith et al., 1994), DR3/WSL-1/Apo-3/TRAMP/LARD (Chinnaiyan et al., 1996), TRAIL receptors DR4/TRAIL-R1 (Pan et al., 1998a), DR5/TRAIL-R2/KILLER (Screaton et al., 1997), and

DR6 (Pan et al., 1998a), can bind a specific ligand at the cell surface. This binding induces receptor oligomerization. In the case of Fas and TNFR1, this results in the recruitment and autoproteolytic activation of procaspase-8 associated with the DISC (death-inducing signaling complex) via interactions between the death domains of receptor and FADD, and the death effector domains of FADD and procaspase-8 (Medema et al., 1997). The autoactivated caspase-8, consisting of two subunits each of p18 and p10, is released from the DISC into the cytosol to initiate caspase activation events.

In the second example, a mitochondrially located protein complex named the 'apoptosome', can regulate the activation of procaspase-9 (Li et al., 1997). Procaspase-9 is recruited to Apaf-1 by a cytochrome c/dATP dependent mechanism. This leads to the autoproteolytic processing of procaspase-9 via association with Apaf-1 oligomers (Srinivasula et al., 1998) . Bcl-xL can bind to Apaf-1 to prevent it from forming oligomers, thereby preventing the autocatalytic activation of procaspase-9 (Srinivasula et al., 1998). As well, Bcl-xL/Bcl-2 prevents release of cytochrome c from mitochondria in response to a death signal (Li et al., 1997; Pan et al., 1998b).

The two caspase activation complexes have been linked via an activation cascade and amplification pathway (Li et al., 1998; Luo et al., 1998). Ligand binding to the death receptor can induce the activation of caspase-8 associated with DISC (Li et al., 1998). The activated caspase-8 can specifically cleave Bid, a pro-apoptotic member of the Bcl-2 family of proteins, at its N-terminal caspase recognition site (Li et al., 1998; Luo et al., 1998). The truncated Bid moves to the mitochondrial outer membrane and induces the release of cytochrome c from the organelle (Luo et al., 1998). The resulting activation of caspase-9 can further amplify the caspase activation cascade by cleaving and activating the downstream effector caspases such as caspase-3. The connection between these two caspase activation complexes represents the communication of death signals from the cell surface to mitochondria.

In addition to its location in the mitochondrial outer membrane, Bcl-2 /Bcl-xLis also located at the endoplasmic reticulum (ER). An ER located apoptotic regulatory protein complex has also been proposed (Ng et al., 1997; Ng and Shore, 1998). In this protein

complex, Bap31, an ER integral membrane protein, has been found to associate with Bcl-2/Bcl-xL, procaspase-8, and a Ced-4-like adapter in co-transfected 293T cells (Ng et al., 1997; Ng and Shore, 1998). Although the exact role of this ER located protein complex in the regulation of apoptosis needs to be established, Bap31 itself is the target of caspase-8 and other caspases (Ng et al., 1997). Bap31 contains two caspase-8 recognition sites. Inresponse to an apoptotic stimulus such as expression of Adenovirus E1A, Bap31 is cleaved at both sites to generate a p20 fragment (Ng et al., 1997). The proteolytic p20 product of Bap31 can induce apoptosis in 293T cell when ectopically expressed (Ng et al., 1997). These data suggest that the ER located Bap31 protein complex may represent a novel caspase activation complex in the ER which itself is a target of caspase-8.

A common feature of the Bcl-2 family is their ability to form either homo- or hetero-dimers with one another under certain conditions (Chao and Korsmeyer, 1998; Oltvai et al., 1993; Yang and Korsmeyer, 1996). Two motifs named BH1 and BH2 (Bcl-2 homology regions 1 and 2) are highly conserved and exist in all Bcl-2 anti-apoptotic family members, with the exception of Adenovirus E1B 19kDa (19k), which does not have a BH2 motif. 19K is recognized as a functional homologue of Bcl-2 (Rao et al., 1992). Although 19K interacts with several pro-apoptotic members of the Bcl-2 family of proteins, such as Bax, it does not interact with another pro-apoptotic member, Bad, suggesting that 19K has different protein-protein interaction properties compared to Bcl-2/Bcl-xL (Chen et al., 1996).

BH1 and BH2 domains are required for homodimerization and heterodimerization of the Bcl-2 family of proteins (Chao and Korsmeyer, 1998; Oltvai et al., 1993; Yang and Korsmeyer, 1996). The BH1 and BH2 regions are also required for the anti-apoptotic functions of Bcl-2 (Oltvai et al., 1993). Mutation of the conserved glycine residue within the BH1 domain, which is one of the residues forming the hydrophobic groove in Bcl-2, can both inhibit Bcl-2's anti-apoptotic function and block its heterodimerization with Bax (Yin et al., 1994). An equivalent Gly87 to Ala mutation in the BH1 domain of E1B 19kDa also severely affected its anti-apoptotic function and its ability of hetodimerization with Bax (Chen et al., 1996).

A third conserved region, called the BH3 domain, was first defined in Bak for its essential role in heterodimerization between Bak and Bcl-2, and its role in death promotion (Chittenden et al., 1995). Several death agonist members of the Bcl-2 family of proteins, including Bad, Bik, Bim, Bid, Blk, and Hrk, share no sequence homology with other Bcl-2 family members outside of the BH3 region, indicating that the BH3 domain is required and sufficient for their pro-apoptotic activity. The BH3-only pro-apoptotic Bcl-2 family of proteins can form heterodimers with anti-apoptotic family members but can not form homodimers, suggesting that the BH3 domain alone is sufficient for the heterodimerization. Taken together, the BH3 domain has two functions. One is that it mediates the interaction with anti-apoptotic members. The other is that it is also required for the apoptotic induction function of these BH3-only pro-apoptotic members and in this regard has been termed the 'Killing Domain'.

The fourth conserved domain of the Bcl-2 family of protein, BH4, was defined recently. It is located at the N-terminus of some anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Ced-9 (Huang et al., 1998). This domain has been implicated in protein-protein interactions. Several proteins including Ced-4 (Huang et al., 1998), Apaf-1 (Pan et al., 1998b), calcineurin (Shibasaki et al., 1997), and Raf-1 (Wang et al., 1994) interact with Bcl-2 through the BH4 domain. The BH4 deletion mutant of Bcl-2 has been reported to lose its anti-apoptosis function (Huang et al., 1998).

Although E1B 19kDa can functionally replace Bcl-2 in many contexts, it may operate somewhat differently than Bcl-2. It interacts with Bax, but not with Bad (Chen et al., 1996). Unlike Bcl-2, E1B 19kDa expression can disrupt FADD filament formation and cause FADD and FLICE to relocalize to membrane and cytoskeletal structures (Perez and White, 1998). The cellular distribution of 19K is also different than that of Bcl-2 (Rao et al., 1996; White, 1996). And finally, the domain structure of 19K includes BH1 and overlapping BH3/BH4, but is devoid of BH2. Here, we demonstrate that these differences are manifested in the functional difference that 19K does not interact with Bcl-xL and Bap31. Swapping the BH3/4 domain of 19K with that of Bcl-2, however, permits such interactions, indicating that Bcl-2 BH3 and 19K BH3/4 are functionally distinct.

## *4.3 Materials and Methods*

### 4.3.1 Constructs and Expression Vectors

Standard recombinant polymerase chain reaction methodology was employed to insert oligonucleotides encoding the HA epitope,(M)AYPDYVPDYAV, or Myc epitope, EQKLISEEDL, at the 3'-end of the protein-coding sequence of E1B 19kDa and Bcl-xL cDNA respectively; PCR was also employed to make the deletion and domain swapping constructs. cDNAs encoding human Bcl-xL and Adenovirus serotype 5E1B 19kDa HA and its domain swapping mutants, were cloned into the Rs/RSV vector (Pharmacia Biotech Inc.) carrying a neomycin resistance gene. Bap31 Flag cDNA was cloned into the PCDNA3.1A vector (Invitrogen). The authenticity of all constructs was verified by DNA sequencing.

### 4.3.2 Cell Culture and Stable Line Selection

CHO LR73 fibroblasts were maintained in α-MEM supplemented with 10% fetal bovine serum and streptomycin sulfate/penicillin (100 U/ml). 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and streptomycin sulfate/penicillin (100 U/ml). The cells were grown in a humidified 5% CO2 atmosphere at 37°C. To establish stable cell lines, each appropriate plasmid was transfected into CHO cells. Stable transfectants expressing high levels of protein encoded by transfected plasmid were isolated after a two week selection period in the presence of G-418(800 μg/ml) and characterized by Western analysis of the cell lysates .

### 4.3.3 Cell Viability Assays

Human CHO cells stably expressing the cDNA encoding neomycin resistance (neo) or E1B 19kDa and its mutants were cultured in a-MEM supplemented with 10% FBS and 100 U/ml streptomycin and penicillin. After reaching 80% confluence in a 12-well plate, cells were treated with 20 mg/ml of vinblastine in the culture medium. The treated cells were

collected at 77 hour after drug application and assessed for viability by trypan blue exclusion. The percentage of cells that excluded trypan blue was determined in two independent experiments, and the results were averaged.

### 4.3.4 Transient Transfections and Co-immunoprecipitations

293T cells were seeded in 60 mm culture plates and transfection performed when the cells reached 50-60% confluency. The cells were transfected by the Lipofectamine Plus method (GIBCO) with 5 μg of selected plasmids. Approximately 26 hours after transfection, the cells were washed in PBS and lysed in 0.5 ml lysis buffer/60 mm culture plate (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetate, 0.5% vol/vol IGEPAL, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). After centrifugation at 11,000 g, the supernatants were incubated with 30 μl of a 1:1 slurry of protein A Sepharose4B for 1 hour at 4°C. The Sepharose was removed and the supernatants incubated with the first antibody at 4°C overnight. 20 μl of a 1:1 slurry of protein G Sepharose was added after the overnight incubation. After 1 hour at 4°C, the beads were recovered, washed, and boiled in SDS sample buffer. After SDS-PAGE and transfer to nitrocellulose, blots were developed with either mouse anti-Myc 9E10 antibody (BAbco, Berkeley, CA), mouse anti-HA 12CA5 antibody (BAbco, Berkeley, CA), or mouse anti-FLAG M2 antibody (SIGMA St.Louis), respectively.

### 4.3.5 Western Blotting

Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the appropriate first antibody. Following incubation with secondary antibody conjugated to peroxidase, the blot was developed with ECL(Amersham) according to the manufacturer's instructions.

### 4.3.6 Immunocytochemistry

CHO LR73 cells expressingE1B 19kDa/Bcl2BH3-HA orE1B 19kDa-HA were grown on 12 well chamber slides and fixed in 3.7% formaldehyde/PBS for 20 min. Permeabilization of cells was performed in 0.2% Triton X-100/ PBS for 5 min. The cells were then incubated with mouse anti-HA.11 for 60 min at room temperature to detect the HA tagged E1B 19kDa and its chimeric proteins. After PBS (containing 0.1% Tween 20) washes, cells were incubated with Rhodamine-labeled secondary antibodies and viewed with an immunofluoresence microscope.

## *4.4 Results and Discussion*

AdenovirusE1B 19kDa is a functional homologue of Bcl-2. Early studies showed that 19K interacts with Bax (Chen et al., 1996; Han et al., 1996). Unlike other anti-apoptotic members of the Bcl-2 family, however, 19K does not interact with Bcl-2/Bcl-xL or Bad (Chen et al., 1996), nor does E1B 19kDa interact with Bap31 (unpublished). These results raised the possibility that 19K has distinct features from Bcl-2/Bcl-xL regarding its anti-apoptotic mechanism. This difference may be caused by 19K structural differences compared to Bcl-2/Bcl-xL. For example, 19K does not have a BH2 domain, whereas all the other anti-apoptotic members of Bcl-2 family do. The homology of the BH3 domain in 19K is also low (Figure 4-1, page 69). Using a domain swapping strategy, we have made a set of 19K chimeric proteins in which the individual BH domains from Bcl-2 have been introduced into 19K .(Figure 4-1, page 69). A transient transfection assay was used to assess the ability of these E1B 19kDa chimeras to interact with Bap31 (Figure 4-2, page 70)and Bcl-xL (Figure 4-3, page 71)

As illustrated in Figure 1B, cDNAs encoding three chimeric 19K proteins were constructed: one in which the BH1 domain was replaced with that of Bcl-2, another in which the Bcl-2 BH2 domain was inserted at a position relative to endogenous 19K BH2 that reflects their relative positions in Bcl-2, and a third in which the 19K overlapping BH3/4 domain was replaced with Bcl-2 BH3. The chimeric proteins, as well as wild-type 19K, were tagged at the NH2-terminus with an HA epitope, and the respective cDNAs incorporated into RcRSV vectors and expressed by transient co-transfection of 293T cells with a pCDNA3.1A vector encoding Flag epitope-tagged Bap31. The ability of the 19K proteins to associate with Bap31-Flag was determined by immunoprecipitating Bap31 from cell lysates with mouse anti-Flag antibody, resolving the precipitate by SDS PAGE, and probing blots of these gels with mouse anti-HA. The resulting HA signals were well resolved from the Ig light chain that was also detected by secondary anti-mouse IgG. As shown in Figure 2A(Figure 4-2, page 70), the expression levels of the 19K proteins were variable and, therefore, the relative amounts that associated with Bap31 were assessed by comparing the levels in the Bap31 precipitates with the total expression levels of the 19K proteins. Both wild type 19K and 19K harboring the Bcl-2 BH1 domain were undetectable

**Figure 4-1 Schematic representation of the E1B 19kDa chimeric proteins and an alignment of the BH4 domains from different Bcl-2 family members.**

(A). Alignment of the BH4 regions from different Bcl-2 family members, as well as from the E1B 19kDa/Bcl-2BH3 chimeric protein. (B). Schematic representation of the E1B 19kDa chimeric proteins carrying BH1, BH2, and BH3 domains from Bcl-2.

Figure 1A

| | |
|---|---|
| Bcl-2 | D N R E I V M K Y I H Y K L S Q R G Y E W |
| Bcl-xL | S N R E I V V D F L S Y K L S Q K G Y S W |
| E1B 19kDa | F W R F L W G S S Q A K L V C R I K E D Y |
| Mcl-1 | Q S L E I I S R Y L R E Q A T G A K E T K |
| A1 | H I H S L A E H Y L Q Y V L Q V P A F E S |
| E1B 19kDa/Bcl-2BH3/BH4 | F W R F L V P P V V H L T L R Q A G D D F |

Figure 1B

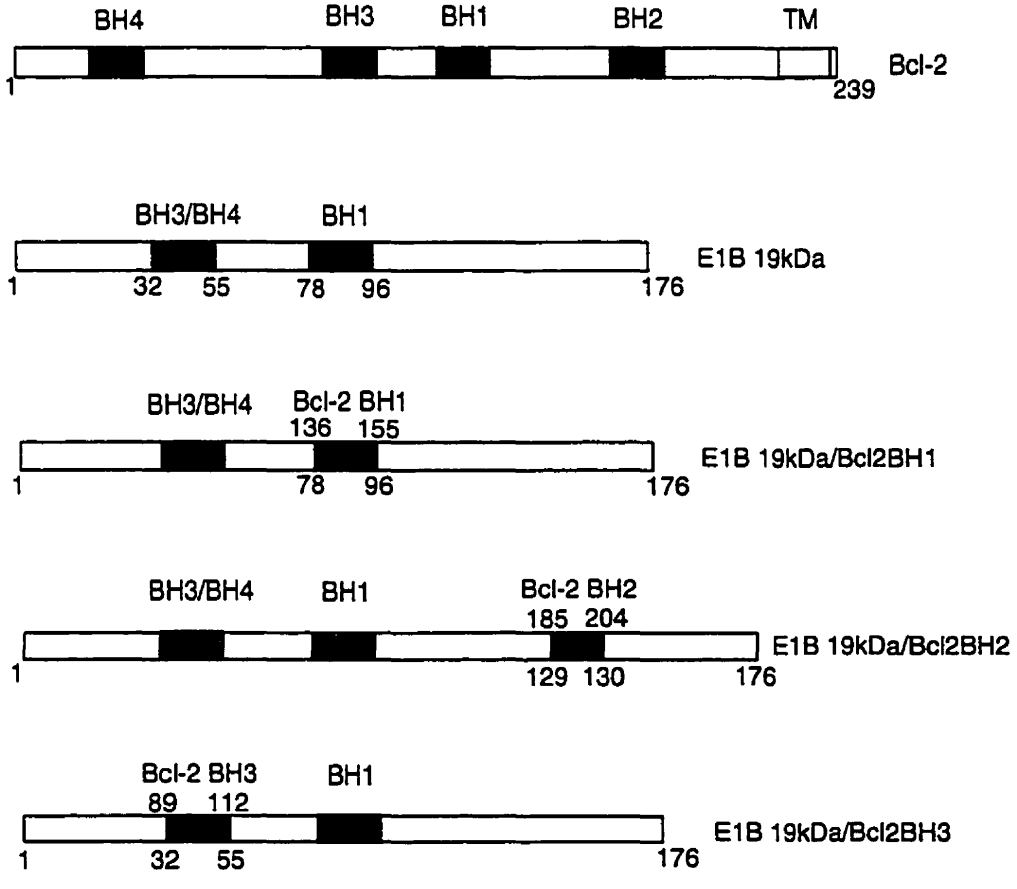


Figure 4-2 Conversion of E1B 19kDa into a Bap31 binding protein.

(A). 293T cells were transiently transfected with vector expressing Bap31-Flag together with E1B 19kDa-HA, E1B 19kDa/Bcl2BH1-HA, E1B 19kDa/Bcl2BH2-HA, and E1B 19kDa/Bcl2BH3-HA, respectively. At 24 hours after transfection, cells were collected and lysed. Co-immunoprecipitation was carried out as described in Materials and Methods with mouse anti FLAG antibody. The precipitated samples and cell lysates were resolved by SDS-PAGE and transfered to nitrocellulose membranes. The membranes were blotted with mouse anti HA antibody and visualized by ECL. Top panel: cell lysates probed with anti HA antibody. Middle panel: cell lysates probed with anti FLAG antibody. Bottom panel: anti FLAG immunoprecipitation samples probed with anti HA antibody. Arrow indicates IgG light chain.

(B). Percentage of E1B 19kDa chimeric proteins co-immunoprecipitated with anti FLAG antibody. NIH IMAGE analysis software is used for this analysis.

Figure 2A

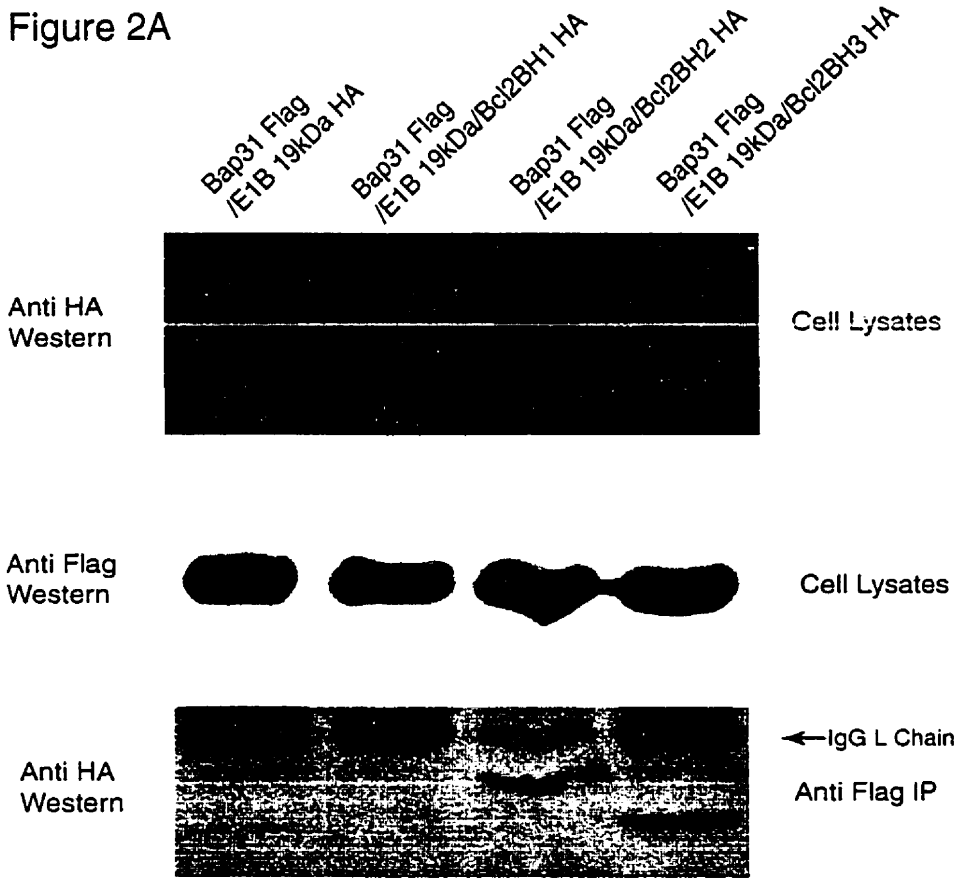



Figure 2B

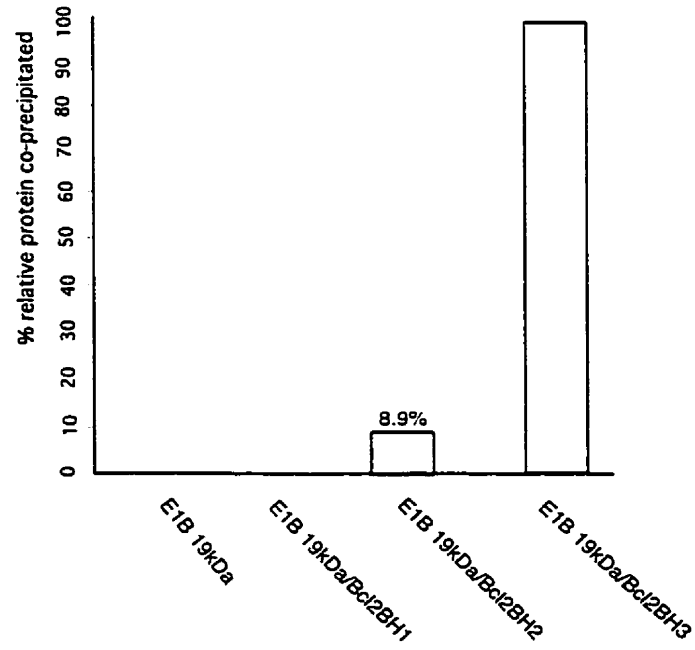
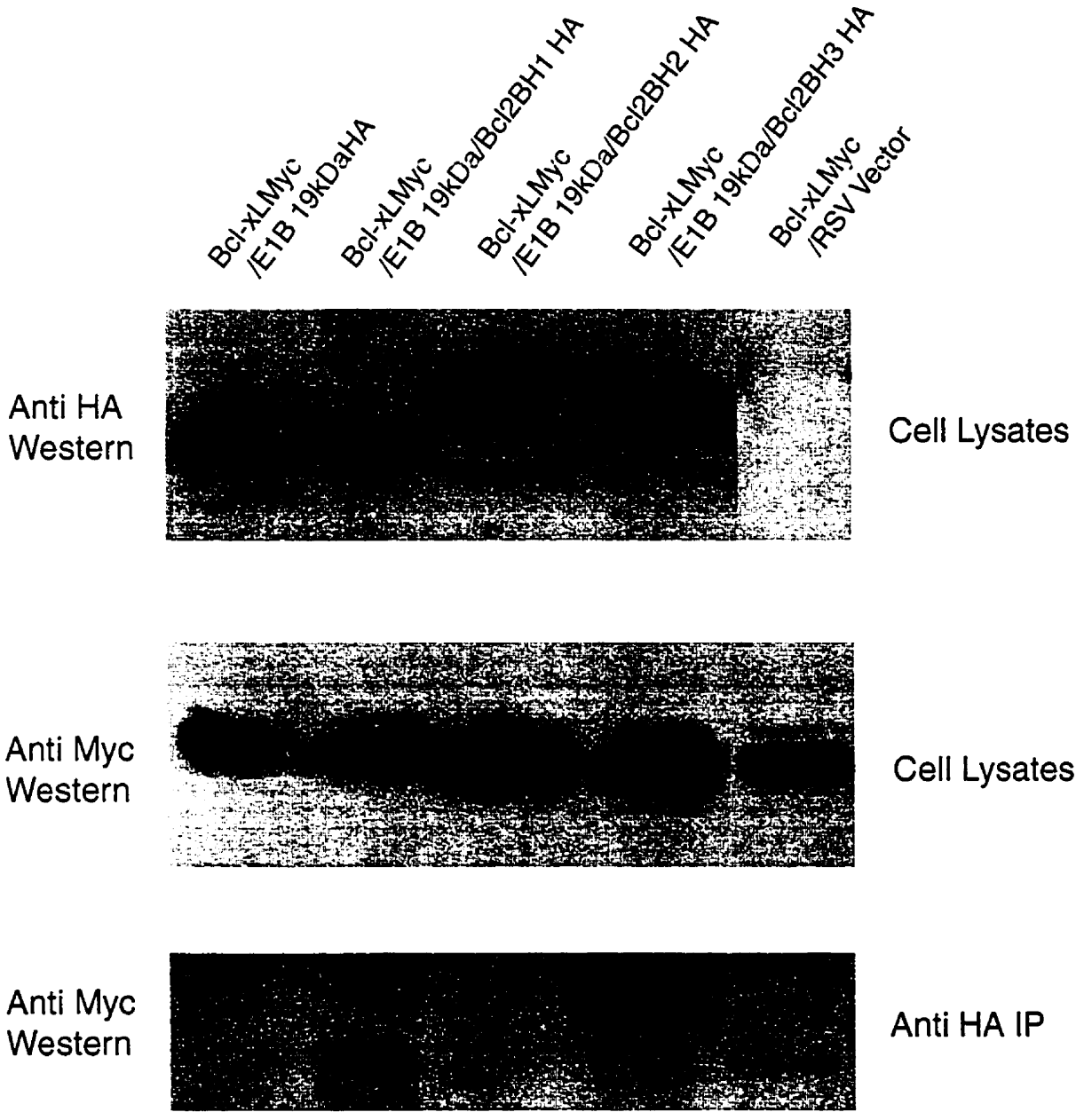


Figure 4-3 Conversion of E1B 19kDa into a Bcl-xL binding protein.

293T cells were transiently transfected with vector expressing Bcl-xL-MYC together with E1B 19kDa-HA, E1B 19kDa/Bcl2BH1-HA, E1B 19kDa/Bcl2BH2-HA, and E1B 19kDa/Bcl2BH3-HA, or empty expression vectors respectively. At 24 hours after the transfection, cells were collected and lysed. Co-immunoprecipitations were carried out as described in the Materials and Methods with mouse anti HA antibody. The precipitated samples and cell lysates were resolved by SDS-PAGE and transfered to nitrocellulose membranes. The membranes were blotted with mouse anti MYC antibody and visualized by ECL. Top panel: cell lysates probed with anti HA antibody. Middle panel: cell lysates probed with anti MYC antibody. Bottom panel: anti HA immunoprecipitation samples probed with anti MYC antibody.


Bcl-xLMyc /E1B 19kDaHA
Bcl-xLMyc /E1B 19kDa/Bcl2BH1 HA
Bcl-xLMyc /E1B 19kDa/Bcl2BH2 HA
Bcl-xLMyc /E1B 19kDa/Bcl2BH3 HA
Bcl-xLMyc /RSV Vector
Anti HA Western
Cell Lysates
Anti Myc Western
Cell Lysates
Anti Myc Western
Anti HA IP

in Bap31 precipitates, and 19K containing Bcl-2 BH2 exhibited only weak association. In contrast, 19K containing Bcl-2 BH3 showed a strong association with Bap31(Figure 4-2, page 70). Similar results were obtained for the ability of the various 19K proteins to associate with Bcl-xL. Again, the only 19K protein that appeared to a significant degree in the Bcl-xL precipitates was 19K harboring the Bcl-2 BH3 domain (Figure 4-3, page 71). When stably expressed in CHO cells, wild type 19K and the 19K/Bcl-2BH3 chimeric protein both protected these cells against apoptosis induced by the drug vinblastine, commensurate with the respective expression levels of the two proteins (Figure 4-4, page 73). Swapping the endogenous BH3/4 domain of 19K with Bcl-2 BH3, therefore, retains 19K function.

Although E1B 19kDa has been classified as a anti-apoptotic member of the Bcl-2 family, amino acid alignments and localization studies of E1B 19kDa show distinct features in comparison to Bcl-2 (Rao et al., 1996; White, 1996). Bcl-2 is anchored via its carboxyl-terminal hydrophobic region to the outer membranes of mitochondria, nuclei, and the endoplasmic reticulum leaving the bulk of the protein facing the cytoplasm (Chao and Korsmeyer, 1998; Hockenbery et al., 1990; Nguyen et al., 1993). In contrast, E1B 19kDa is found to associate with the nuclear lamina as well as the cytoplasmic and nuclear membranes (White and Cipriani, 1990). Functionally, E1B 19kDa has been found to block TNF induced apoptosis whereas Bcl-2 is ineffective (White, 1996). E1B 19kDa has also been shown to activate the JNK and c-JUN mediated transcription pathway which, together with upstream MEKK1 and MKK4, has been implicated in apoptosis regulation (See and Shi, 1998). These data suggest that E1B 19kDa may use multiple mechanisms to exert its anti-apoptotic function. 293T cells transiently transfected with vectors expressing 19K or 19K/Bcl-2BH3 were analyzed by immunofluorescence (Figure 4-5, page 74), and while not studied in detail, the results suggest primarily a cytoplasmic distribution for both proteins, with 19K/Bcl-2BH3 exhibiting an enhanced predisposition for the perinuclear / nuclear envelope region of the cell. CHO cells stably expressing an E1B 19kDa/Bcl2BH3 chimeric protein retained anti-apoptotic activity in comparison with cells expressing wild type E1B 19kDa (Figure 4-4, page 73).

Figure 4-4 Functional analysis of the E1B 19kDa/Bcl2BH3 chimeric protein.

CHO cells stably expressing the neomycin resistance gene (Neo), E1B 19kDa or E1B 19kDa/Bcl2BH3, respectively were treated with 20 mg/ml of vinblastine in the culture medium. The treated cells were collected at 60 hours after drug was applied and assessed for viability by trypan blue exclusion. The Western analysis shows the level of E1B 19kDa and E1B 19kDa/Bcl-2BH3 proteins in the stably transfected CHO cells.

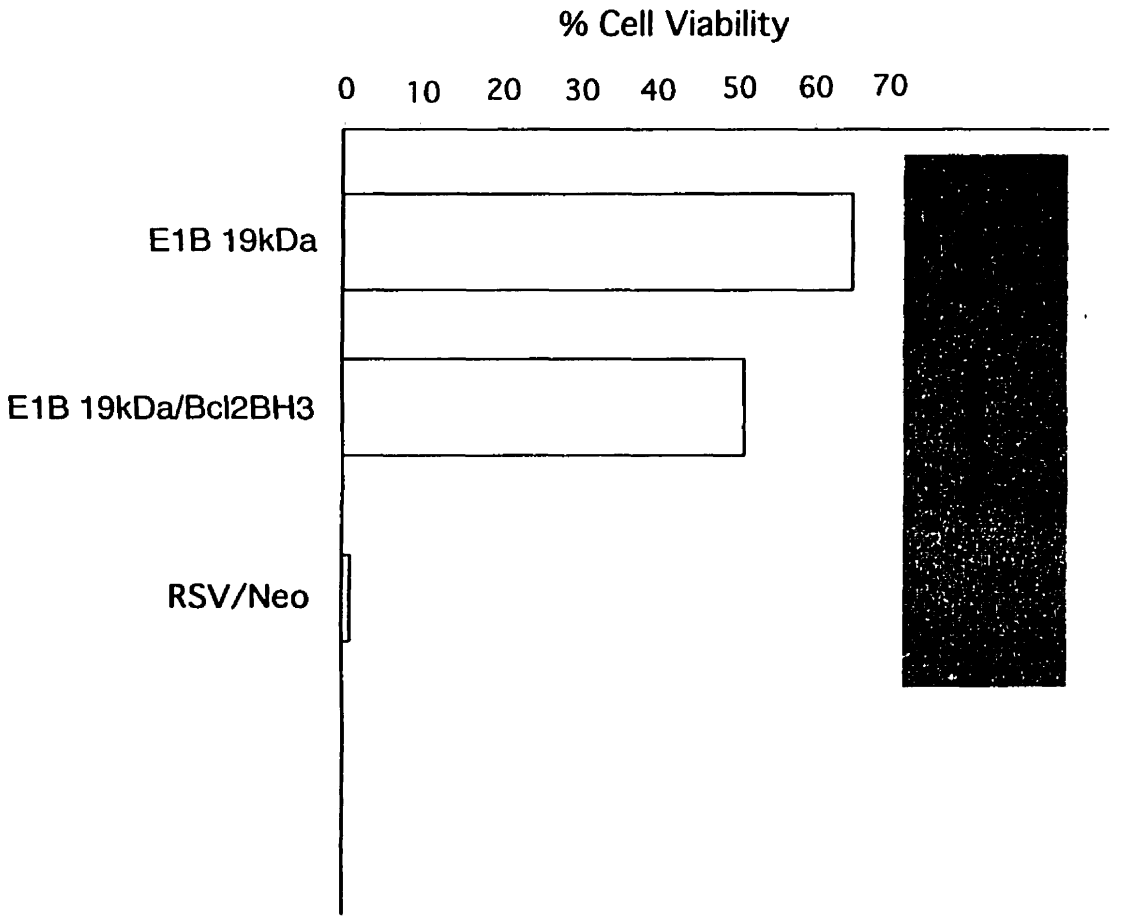

% Cell Viability
0
10
20
30
40
50
60
70
E1B 19kDa
E1B 19kDa/Bcl2BH3
RSV/Neo

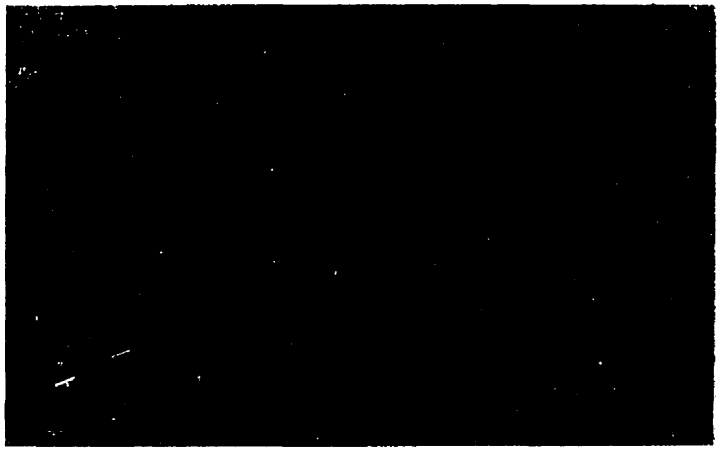
**Figure 4-5 Subcellular distribution study of the E1B 19kDa/Bcl2BH3 chimeric protein and E1B 19kDa by immunofluoresence microscopy.**

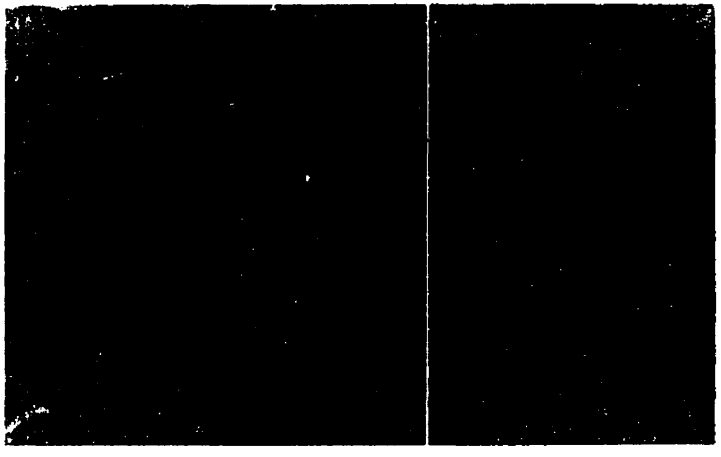
Briefly, E1B 19kDa/Bcl2BH3 and the E1B 19kDa expression vectors were individually transiently transfected into CHO LR73 cells. The transfected CHO cells were grown on 12 well chamber slides and fixed as described in the Materials and Methods. Cells were then incubated with primary antibody as described in Materials and Methods. After PBS washes, cells were incubated with Rhodamine-labeled secondary antibodies and viewed by immunofluoresence microscopy.

E1B 19kDa/Bcl-2BH3

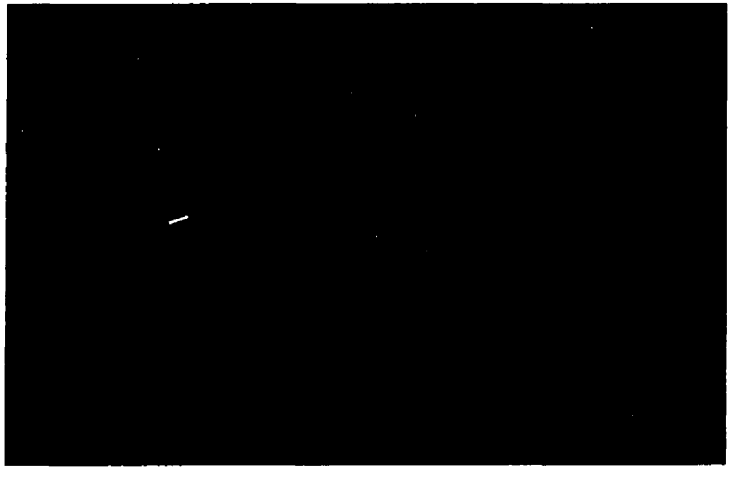
E1B19kDa

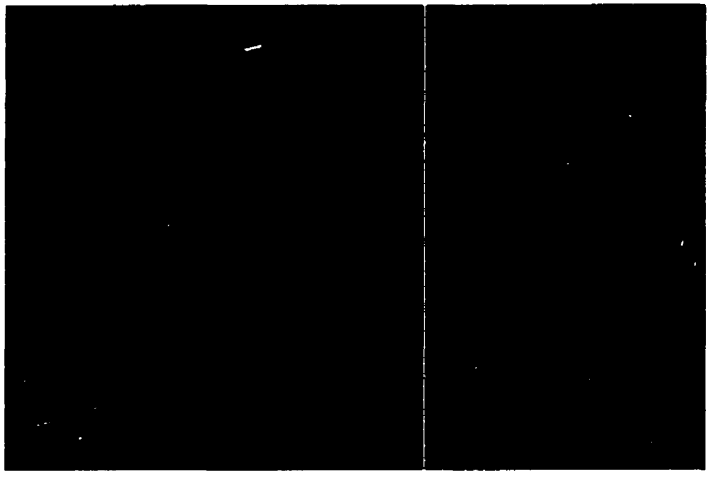
Phase





Rhodamine-labeled





The BH3 domain is involved in the heterodimerization process between different Bcl-2 family members and the BH4 domain is essential for the interaction between Bcl-2/Bcl-xL and Ced-4 (Chittenden et al., 1995; Huang et al., 1998). Our alignment shows that E1B 19kDa has a weak BH3/BH4 domain (Figure 4-1, page 69). The E1B 19kDa/Bcl2BH3 chimeric protein can interact with Bap31 and Bcl-xL while the wild type E1B 19kDa does not (Figure 4-2, page 70)(Figure 4-3, page 71). These results suggest that the BH3 of Bcl-2 and BH3/4 of E1B 19kDa have distinct protein-protein interaction properties and that E1B 19kDa may use multiple mechanisms to regulate apoptosis, depending on its subcellular location and the availability of other regulators. Some of the mechanisms used by E1B 19kDa will be different from that of Bcl-2. One example is that E1B 19kDa can block the FADD and FLICE mediated apoptosis pathway by sequestering FADD in a FLICE dependent manner (Perez and White, 1998). Bcl-2 does not function through this pathway.

In summary, we demonstrated that the BH3 domain of Bcl-2, when inserted into the homologous region in E1B 19kDa, confers the Bcl-2 interaction properties with Bap31 onto E1B 19kDa. Other conserved BH regions of Bcl-2 are either not required or dispensable in these protein-protein interactions. These results show that the BH3 domain of Bcl-2/Bcl-xL has an important role in mediating Bcl-2/Bcl-xL and Bap31 interactions. It also demonstrates that the BH3 domain of Bcl-2 facilitates different protein-protein interactions compared to the BH3 domain of E1B 19kDa.

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# CHAPTER 5

## General Discussion

Apoptosis is a genetically controlled and evolutionarily conserved process that is essential for both embryogenesis and homestasis. It has been one of the fastest growing research fields in the past decade.

Regulation of apoptosis is fundamentally important in multicellular organisms. It is involved in the necessary elimination of certain neuronal cells during development, cell death between the digits during development in vertebrates, and the elimination of self-reactive pre-T and pre-B cells during lymphocyte maturation. Inappropriate activation or inactivation of the apoptosis program can cause a diverse collection of genetic and acquired diseases such as cancer, neurodegenerative diseases and AIDS. The research aimed at defining the regulatory mechanisms of apoptosis has been and will continue to be a focus for pharmaceutical, medical, and biological research. It will greatly expand our knowledge of the apoptosis pathway and facilitate the treatment of apoptosis related diseases.

Despite of the progress made in apoptosis research, our knowledge of the functional mechanism of Bcl-2 family proteins remains largely unknown. Qttempts to isolate Bcl-2 interacting proteins have led to the characterization of more than a dozen Bcl-2 family members which are either pro- or anti-apoptotic, suggesting that the interactions between different pro- and anti-apoptotic Bcl-2 family members are important for their regulatory function.

The adenovirus E1B 19kDa protein was characterized as a viral homologue of Bcl-2. E1B 19kDa is an adenovirus early gene product that has been found to protect against cell death induced by viral infection, facilitating efficient virus replication by preventing premature cell death. At the time the research described in chapter 2 was conducted, little was known about the function of the Bcl-2 family of proteins. Although the survival promoting activity of Bcl-2 has been examined against the effects of multitudes of death stimuli, such as chemotherapeutic drugs and ionizing radiation, the effect of E1B 19kDa on the apoptotic effects of these stimuli had not been examined extensively. To characterize the anti-apoptotic function of E1B 19kDa and to understand the functional differences between E1B 19kDa and Bcl-2, it was essential to compare the ability of E1B 19kDa and

Bcl-2 to block apoptosis triggered by different death stimuli. Chapter 2 describes our attempt to address this question.

Hygromycin B, an aminoglycoside antibiotic, is widely used to establish stable mammalian cell lines that carry a bacterial gene conferring resistance to the drug. We know very little about the mechanism by which the sensitive cells die in response to hygromycin B. During my studies, I found that hygromycin B kills cell by apoptosis ( Figure 1, Chapter 2, page 57-56). The apoptosis induced by hygromycin B shows all the classic features, including cell shrinkage, membrane blebbing, nuclear condensation, and internucleosomal fragmentation of DNA (Figures 1 and 2, Chapter 2, page 57-56). Apoptosis can be induced independently of p53, both during the normal developmental process and in response to a variety of genotoxic agents. Hygromycin B falls into this p53-independent category. The apoptosis induced by hygromycin B can occur in the absence of wild-type p53 (Figure 5, Chapter 2, page 57-58). Paradoxically, however, both cycloheximide and actinomycin D (inhibitors of protein and RNA synthesis, respectively) blocked the apoptotic response to hygromycin B, despite the fact that hygromycin B itself is an inhibitor of protein synthesis. Therefore, it is not clear whether the mechanism of induction of apoptosis by hygromycin B relates to its effect on protein synthesis or on some other pathway. Data from Chapter 2 showed that both E1B 19kDa and Bcl-2 can block the apoptosis triggered by hygromycin B (Figure 4, Chapter 2, page 57-58). Bcl-2 is a well known suppressor of apoptosis. E1B 19kDa, on the other hand, is a viral suppressor of apoptosis which has evolved specifically to counter the cytotoxic effects of adenovirus E1A gene expression, thus allowing E1A-induced host DNA synthesis to proceed. In spite of the functional similarity between E1B 19kDa and Bcl-2 shown during adenovirus infection and BRK (baby rat kidney) cell transformation, the extent of the similarity in the ability of Bcl-2 and E1B 19kDa to suppress apoptosis has not been fully compared. The fact that E1B 19kDa also counters hygromycin B mediated cell death suggests that, like Bcl-2, it too may confer protection against apoptosis in a variety of circumstances.

One important function of Bcl-2 is to form heterodimers with certain pro-apoptosis members such as Bax and Bad. The ratio of pro- and anti-apoptotic proteins in the cell forms a setpoint, which decides whether the cell should die or survive after receiving a

death signals. When the cell contains more of the free form of pro-apoptotic members such as Bax, its apoptosis pathway can be turned on. When Bcl-2 or other anti-apoptotic members dominate, the cell survives. Taken together, the heterodimerizing partners consisting of pro- and anti-apoptotic members of the Bcl-2 family establish a set point within the cell that determines whether or not the cell will ultimately respond to a death signal.

Bad, a more distant member of the Bcl-2 family that belongs to the pro-apoptotic subfamily, is a direct regulator of the Bax/Bcl-2 setpoint (Yang et al., 1995). The function of Bad is to compete with Bax for Bcl-2 heterodimerization, freeing Bax from the Bcl-2/Bax heterodimer such that the apoptosis setpoint is directed towards apoptosis (Yang et al., 1995). Although it is not presently known how the setpoint controls the apoptotic program in the cell, two possible models has been proposed regarding the regulation of the Bcl-2 and Bax setpoint. First, Bcl-2 and Bax function independently to interact with survival- and death-promoting molecules, but could neutralize each other's function through heterodimerization. Another model is that the Bax homodimer could be the active component, which triggers apoptosis in the cell. In this case, Bcl-2 form heterodimers with Bax to protect the cell from apoptosis. In either case, the level of Bax and Bcl-2 heterodimer can act as a setpoint in the cell to determine whether the cell should live or die.

The data presented in Chapter 3 shows the investigation of the functional similarities and differences between E1B 19kDa and Bcl-2 regarding their ability to regulate the apoptotic setpoint in the cell. The BH1 domain of Bcl-2 is critical for Bax/Bcl-2 heterodimerization (Oltvai et al., 1993). Gly145 in the BH1 domain of Bcl-2 is conserved in all Bcl-2 family members (Yin et al., 1994). A relatively conservative change of a glycine to alanine at residue 145 of the Bcl-2 BH1 domain results in loss of both Bcl-2 function and its ability to heterodimerize with Bax (Yin et al., 1994). The sequence similarities between E1B 19kDa and Bcl-2 are largely restricted to two short BH domains, the BH1 and BH3 (later on, in Chapter 4, we showed that E1B 19kDa also contains a BH4 which overlaps with its BH3 domain). I found that the BH1 domain of E1B 19kDa contributes to its anti-apoptotic function and its ability to interact with Bax as well (Figures 4 and 5, Chapter 3, page 58-24224). An E1B 19kDa mutant, which carries a glycine 87 to alanine mutation in the BH1

region, failed to interact with Bax (Figure 4, Chapter 3, page 58-24224). This result suggests that the same conserved residue in the BH1 domain that is critical for the Bcl-2 and Bax interaction is also important for the E1B 19kDa and Bax interaction (Figure 4, Chapter 3, page 58-24224). However, in contrast to Bcl-2, we did not detect any interaction between E1B 19kDa and Bad (Figure 6, Chapter 3, page 58-24224). Furthermore, the anti-apoptotic effect of E1B 19kDa was not overcome by enforced overexpression of Bad in transfected cells. This has obvious evolutionary implications for the ability of the virus to evade potential host defense mechanisms, because it permits E1B 19kDa to target Bax while at the same time avoiding disruption of this interaction and re-instatement of the apoptotic program by Bad. This is the first time that E1B 19kDa and Bcl-2 have been shown to act differently in their ability to interact with pro-apoptotic members of the Bcl-2 family. Based on these results, we proposed that E1B 19kDa is a structural and functional homolog of Bcl-2, but has unique features. Comparisons of the functional differences between Bcl-2 and E1B 19kDa will provide an opportunity to correlate function with domain structure. Chapter 4 illustrates my attempt to address this question by dissecting different BH domains from Bcl-2 and swapping them into the corresponding regions of E1B 19kDa.

The studies described in Chapter 4 were conducted after a novel Bcl-2/Bcl-xL associating protein, Bap31, was identified in our lab using a Far Western approach with Bcl-2 as a probe (Ng et al., 1997). Bap31 is an integral membrane protein with three potential transmembrane regions. The 1st and 3rd transmembrane regions contain one and two positively charged residues respectively, suggesting that other partner proteins exist in the Bap31 complex to neutralize these charges (Ng et al., 1997). Based on the studies carried out by Florence Ng in the lab, an ER located apoptotic regulatory protein complex has been proposed (Ng et al., 1997; Ng and Shore, 1998). In this protein complex, Bap31 can associate with Bcl-2/Bcl-xL, procaspase-8, and a Ced-4 like adapter protein in co-transfected 293T cells (Ng et al., 1997; Ng and Shore, 1998). Although the exact role of this ER located protein complex in the regulation of apoptosis needs to be established, Bap31 itself is the target of caspases as it contains two caspase recognition sites (Ng et al., 1997). Upon administration of an apoptotic stimulus, Bap31 can be cleaved at both caspase recognition sites by caspase-8 to give p20 and p10 fragments (Ng et al., 1997). The

proteolytic product of Bap31, p20, can induce apoptosis in 293T cells when ectopically expressed (Ng et al., 1997). Bap31 can also be cleaved by an unidentified ER lumenal caspase at its two caspase cleavage sites located in the membrane spanning regions during apoptosis (Gang Chen, unpublished). These data suggests that the ER located Bap31 protein complex has a regulatory effect on the apoptotic process of the cell and may be involved in the amplification of the caspase cascade inside of the cell, communicating apoptotic signals between the ER and mitochondria.

Although E1B 19kDa is a anti-apoptotic member of the Bcl-2 family, its polypeptide sequences and subcellular localizations are quite different from Bcl-2 (Rao et al., 1996; White, 1996). Bcl-2 harbours C-terminal signal-anchor sequences that target it predominantly to the outer mitochondrial membrane, endoplasmic reticular membrane and the outer nuclear envelope leaving the bulk of the protein facing the cytoplasm (Chao and Korsmeyer, 1998; Hockenbery et al., 1990; Nguyen et al., 1993). E1B 19kDa , however, is found to associate with the nuclear lamina as well as the cytoplasmic and nuclear membranes (White and Cipriani, 1990). E1B 19kDa can block TNF induced apoptosis whereas Bcl-2 has been reported to be not effective (White, 1996). E1B 19kDa has also been demonstrated to have a transcription regulatory function (See and Shi, 1998). It can activate the JNK and c-JUN mediated transcription pathway which has been implicated in apoptosis regulation (See and Shi, 1998). Take together, E1B 19kDa may use many different mechanisms for its anti-apoptotic function. 293T cells transiently transfected with vectors expressing 19K or 19K/Bcl-2BH3 were analyzed by immunofluorescence (Figure 4-5, page 74), and while not studied in detail, the results suggest primarily a cytoplasmic distribution for both proteins. CHO cells stably expressing the E1B 19kDa/Bcl2BH3 chimeric protein has the same anti-apoptotic activity as cells expressing wild type E1B 19kDa (Figure 4-4, page 73).

The BH4 domain is important for the interaction between Bcl-2/Bcl-xL and Ced-4 while the BH3 domain plays role in the heterodimerization process between different Bcl-2 family members (Chittenden et al., 1995; Huang et al., 1998). A weak BH3/BH4 domain has been found when we aligned E1B 19kDa with other Bcl-2 family members (Figure 4-1, page 69). The wild type E1B 19kDa does not interact with Bap31 or Bcl-xL(Figure 4-2,

page 70)(Figure 4-3, page 71). But the E1B 19kDa/Bcl2BH3 chimeric protein can interact with both Bap31 and Bcl-xL(Figure 4-2, page 70)(Figure 4-3, page 71). These results indicate that the BH3 from Bcl-2 and the BH3/4 from E1B 19kDa have different protein-protein interaction properties and that E1B 19kDa may uses different mechanisms for apoptosis regulation, depending on its subcellular locations and the availability of other regulatory proteins. Some of these mechanisms will be different from those used by Bcl-2. The fact that E1B 19kDa can block FADD and FLICE mediated apoptosis whereas Bcl-2 does not function through this pathway is a good example (Perez and White, 1998). These data demonstrated that the BH3 domain of Bcl-2, when inserted into the homologous region in E1B 19kDa, can confer the Bcl-2 interaction properties with Bap31 and Bcl-xL onto E1B 19kDa, suggesting that the BH3 domain of Bcl-2 facilitates different protein-protein interactions compared to the BH3 domain of E1B 19kDa. These results also show that the BH3 domain of Bcl-2/Bcl-xL has an important role in mediating Bcl-2/Bcl-xL and Bap31 interactions.

In summary, my Ph.D. thesis has demonstrated the novel properties of the adenovirus anti-apoptotic protein E1B 19kDa. At the early stages of my work, my research focus was on the study of the anti-apoptotic effects of E1B 19kDa against different apoptotic stimuli. With the rapid progression in the apoptotic research field, my research shifted onto a comparison of the functional mechanism between E1B 19kDa and Bcl-2. This work revealed that E1B 19kDa does have properties unique from Bcl-2/Bcl-xL and other anti-apoptotic members of the Bcl-2 family. Characterization of these distinct properties of E1B 19kDa will be of great help to our understanding of the structure/function relationships between the different members of the Bcl-2 family of proteins.

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