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IDENTIFICATION AND CHARACTERIZATION OF Bcl-2/Bcl-X_L INTERACTING PROTEIN, p28Bap31

by

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Abstract

Programmed cell death (apoptosis) is a fundamental process in multicellular organisms. The human proto-oncogene *bcl-2* functions to suppress apoptosis and promote cell survival. It is the most studied apoptotic regulator, yet the mechanism of action of this protein remains elusive.

We have identified p28Bap31 as a Bcl-2 associating protein which also interacts with Bcl-X_L. p28Bap31 is a polytopic integral membrane protein which resides primarily in the endoplasmic reticulum with an N_{lumen}-C_{cyto} orientation. This orientation exposes the COOH-terminal leucine zipper overlapping with a weak death effector domain, flanked on either side by caspase cleavage sites, to the cytosol.

In the absence of Bcl-2, p28Bap31 is cleaved during apoptosis mediated by E1A generating a 20kDa product, p20Bap31. On the contrary, expression of Bcl-2 suppresses cell death and blocks the proteolytic cleavage of p28Bap31. *In vitro* cleavage studies suggested that p28Bap31 is a substrate for caspase-8, an initiator caspase, but not for effector caspases such as caspase-3. Although it is not known whether the cleavage of p28Bap31 contributes to the cell death program, ectopic expression of p20 can trigger apoptosis which can be rescued by co-expression of Bcl-2. To date, it is not clear how p20 induces apoptosis. However, it is possible that it does so by amplification of the caspase cascade, or by activation of a parallel pathway.

In cotransfected 293T cells, p28Bap31 associates simultaneously with both Bcl-X_L and procaspase-8, presumably depending on the presence of a Ced-4-like adaptor molecule. The direct interaction between p28Bap31 and *C. elegans* Ced-4 suggests that a mammalian Ced-4-like molecule may be a normal constituent of the p28Bap31 protein complex in the ER. It also further supports the idea that p28Bap31 plays a role in the regulation of apoptosis. Moreover, the function of this protein complex may be analogous to the Fas/TNFR1 death-inducing signaling complexes by providing an intracellular site for the recruitment of procaspase-8. The presence of Bcl-2-like proteins may act to prevent the processing and activation of the initiator caspase, thereby inhibiting downstream events culminating in cell death.

Résumé

La mort cellulaire programmée (apoptose) est un processus fondamental chez les organismes multicellulaires. Le proto-oncogène humain *bcl-2* agit en supprimant l'apoptose et en favorisant la survie cellulaire. Bien que *bcl-2* soit le régulateur de l'apoptose le plus étudié, son mécanisme d'action demeure obscur.

Nous avons identifié p28Bap31 en tant que protéine associée à Bcl-2, capable également d'interagir avec Bcl-X_L. p28Bap31 est une protéine membranaire localisée principalement dans le réticulum endoplasmique, exposant sa région N-terminale vers la lumière et son extrémité C-terminale vers le cytoplasme. Une telle orientation permet d'exposer vers le cytosol un domaine "leucine zipper" de même qu'une région de faible homologie au domaine effecteur de la mort "(death effector domain)", entouré de chaque coté par des sites de clivage par les caspases.

En l'absence de Bcl-2, p28Bap31 est clivée durant l'apoptose médiée par E1A, générant ainsi une protéine de 20 kDa, p20bap31. Par contre, l'expression de Bcl-2 supprime la mort cellulaire et bloque le clivage protéolytique de p28Bap31. Des études de clivage réalisées *in vitro* suggèrent que p28Bap31 pourrait constitué un substrat pour la cystéine protéase "caspase-8", une caspase initiatrice, mais pas pour les caspases effectrices telles que la caspase-3. Bien qu'il ne soit pas déterminé si le clivage de p28Bap31 contribue au programme de mort cellulaire, l'expression ectopique de p20 conduit à l'apoptose et ce phénomène peut être bloqué par la co-expression de Bcl-2. Jusqu'à maintenant, le mécanisme par lequel p20 induit l'apoptose n'est pas connu. Il est possible que p20 agisse en permettant l'amplification de la cascade de caspases, ou en activant une voie parallèle conduisant à la mort cellulaire.

Basé sur des études de cotransfection réalisées dans les cellules 293T, p28Bap31 s'associe simultanément avec Bcl-X_L et procaspase-8, association présumément dépendante de la présence d'une molécule adaptatrice de type Ced-4. L'interaction directe entre p28bap31 et Ced-4 provenant de l'organisme *C. elegans* , suggère qu'une molécule homologue à Ced-4 existe chez les mammifères, et qu'elle puisse constituer une composante normale du complexe protéique associé à p28Bap31 dans le réticulum endoplasmique. Ceci supporte également l'idée que p28Bap31 joue un rôle

dans la régulation de l'apoptose. Qui plus est, la fonction de ce complexe protéique pourrait être analogue aux complexes de signalisation de la mort cellulaire médiée par Fas/TNFR1, en fournissant un site intracellulaire pour le recrutement de la procaspase-8. La présence de protéines de type Bcl-2 pourrait agir en prévenant le clivage et l'activation de la caspase initiatrice, inhibant ainsi les évènements situées en aval culminant à la mort cellulaire.

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Finally and most importantly, I would like to dedicate this thesis to my parents for their unconditional love and support. I am proud to be their child and I hope I made them proud, too.

Preface

This thesis was organized according to the guidelines instructed by the Faculty of Graduate Studies and Research of McGill University. In accordance with the regulations concerning manuscript-based formats. the following paragraphs are reproduced in full:

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.

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Chapter II: was published in J. *Cell Biol.* (1997) **139**:327-338. p28Bap31, a Bcl-2/Bcl-X_L- and procaspase-8-associated protein in the endoplasmic reticulum. Ng, F. W. H., Nguyen, M., Kwan, T., Branton P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. This work was initiated by myself. Mai Nguyen conducted the transient transfection experiments and the nuclei staining shown in figure 7. She also performed *in vitro* targeting experiments shown in Figure 3. Don Nicholson conducted the *in vitro* cleavage of p28Bap31 shown in Figure 5A. James Cromlish conducted the HPLC experiments shown in Figure 2B. Tony Kwan did the amino acid sequence alignment shown in Figure 2D. Philip Branton provided the adenovirus for the initial infection assay. Gordon Shore provided experimental input and assisted in the preparation of the manuscript. I generated the rest of the data and experimental reagents.

Chapter III: is in press in *J. Biol. Chem.* (1998). Bcl-X_L cooperatively associates with the Bap31 complex in the endoplasmic reticulum, dependent on procaspase-8 and Ced-4 adaptor. Ng, F. W. H., and Shore, G. C.

Chapter IV: will be submitted for publication in 1998. p28Bap31 is an endoplasmic reticulum membrane protein which forms homodimers/-oligomers *in vivo*. Ng, F. W. H., Dahan, S., Bergeron, J. J., and Shore, G. C. Sophie Dahan conducted the immunogold labeling experiments shown in Figure 2 and Table 1. John Bergeron provided antibody against calnexin and purified ER fractions. I was responsible for the generation of the antibodies, DNA constructs and most of the data reported in this chapter.

TABLE OF CONTENTS

List of Figures

List of Tables

Chapter I
Introduction

1. Overview of Programmed Cell death

1.1 History

Physical, chemical, and toxic injury to cells can create environments too hostile for cell survival, and thus cause unexpected death. Such accidents, however, do not account for all the cellular deaths which occur during the life of an organism. Cell death during embryonic development, metamorphosis, the turnover of epithelial and hematopoietic cells, and the development of various lymphocytes are clearly genetically programmed.

Observations made by Kerr over thirty years ago on dying rat hepatocytes caused by hypertrophy and ischaemic injury marked the first breakthrough in programmed cell death research (Kerr, 1965; Kerr, 1971). Prior to his discovery, researchers regarded all cell deaths as necrosis. Necrotic cell death is characterized by the swelling of organelles, rupture of the cell membrane and the release of degraded intracellular debris into the intercellular space which often results in the activation of inflammatory response (Judah et al., 1965). Moreover, when necrosis occurs, it usually involves a large population of cells in the immediate area. Kerr's studies revealed a novel set of cellular events which signified a new class of cell death process displaying characteristics distinct from necrosis. These included the reduction of cell volume, the blebbing of the plasma membrane, progressive perinuclear chromatin condensation and the formation of membrane bound structures which contained intact organelles as well as compacted chromatin (Kerr et al., 1972). Initially this new type of cell death morphology was named "shrinkage necrosis" (Kerr, 1971). Later it was renamed "apoptosis", a Greek word referring to the falling of leaves from trees (Kerr, 1965; Kerr, 1971; Kerr et al., 1972).

For a long time the apoptotic phenomenon had already been noticed by developmental biologists. However, it was the studies by Kerr and colleagues which finally discriminated apoptosis from necrosis and conveyed the idea that apoptosis was involved in tissue kinetics (Kerr et al., 1972). They also indicated that the occurrence of apoptosis was programmed rather than random (Kerr et al., 1972; Saunders, 1966). Unfortunately the significance of this work was not recognized until recently, when researchers finally started to appreciate this sophisticated cell death system. While the terms programmed cell death (PCD) and apoptosis are often used interchangeably, programmed cell death refers to the mechanism by which the unwanted cells are eliminated, while apoptosis describes the morphological changes which occur in cells undergoing programmed cell death.

1.2 Characteristics of Apoptosis

In general, apoptosis can be divided into four stages. These are the commitment phase, the execution phase, the engulfment phase and finally the degradation phase. Although the length of the commitment phase of cells undergoing programmed cell death can range from a few hours to days, the execution of apoptosis is generally rather rapid. Progression from the first physical signs of apoptosis to the phagocytosis of apoptotic corpses is usually completed in under a couple of hours. This rapidity, combined with the fact that apoptosis does not trigger an inflammatory response, may account for the difficulties in bringing the importance of apoptotic programmed cell death to light.

The most studied stage of apoptosis to date has been the execution phase. This is due to the fact that visible physical changes can be detected

in dying cells. The first visible change in cells undergoing apoptosis is the shrinkage of the cell volume, presumably because of water loss (Kerr et al., 1972). The cells then undergo structural changes due to the breakdown of the cytoskeleton. This causes the blebbing of the plasma membrane, or "zeiosis" (Cohen et al., 1992). As a result, membrane-enclosed cytoplasmic contents termed apoptotic bodies, which often contain intact organelles and potential intracellular poisons, are formed (Kerr et al., 1972).

In the nucleus, chromosomal DNA becomes condensed (Kerr et al., 1972). The activated nucleases cleave the condensed chromatin first into large fragments (50-300 kilobases) (Roy et al., 1992) and subsequently to mononucleosome and oligonucleosome sizes, giving rise to the "ladder" pattern seen by DNA electrophoresis (Wyllie, 1980).

In contrast to necrosis, potential intracellular poisons such as proteases, oxidizing molecules and toxic cationic proteins are not released into the intercellular space (Henson and Johnston, 1987). Thus, the activation of inflammatory cells whose activities will lead to the destruction of neighbouring normal cells is avoided. Corpses of the apoptotic cells and apototic bodies are engulfed and phagocytosed by neighbour cells rapidly before they lyse. To date the mechanism by which this phagocytosis occurs is unknown. However, several studies of apoptosis in the immune system proposed three mechanisms by which apoptotic cells are recognized by macrophages. These mechanisms include sugar-lectin interactions (Duvall et al., 1985), phosphatidylserine externalization and recognition (Fadok et al., 1992), and a vitronectin-mediated mechanism (Savill et al., 1990).

1.3 Functions of Programmed Cell Death

Embryonic development and metamorphosis: Long before the discovery of "apoptosis", programmed cell death had already been found to play an essential role during embryonic development (Hammar and Mottet, 1971; Saunders, 1966). There are many developmental processes which show the involvement of apoptotic programmed cell death. For example, during the development of digits in vertebrates, interdigital cell death must occur (Fallon and Cameron, 1977; Hinchliffe and Thorogood, 1974; Pautou, 1974). Unwanted cells must also be eliminated during the regression of Müllerian ducts in mammals and birds which results in the creation of sexual dimorphism (Hinchliffe, 1981; Jost, 1970). It is also essential to abort hyper-reactive pre-T and pre-B cells during lymphocyte maturation in order to avoid autoimmune responses (Cohen et al., 1992). In addition, in order to complete metamorphosis in amphibians, structures like the tails, gills, intestinal epithelium and larval head muscles must be removed prior to the complete transformation into the adult form (Saunders, 1966)

Homeostasis: Genetically programmed cell death is also fundamentally important in maintaining the homeostasis of multicellular organisms. It is a primary goal of a living organism to maintain a steady-state condition. Therefore, aged and damaged cells must be removed to make room for newly divided cells (Kerr et al., 1972) . As discussed below, deregulation of programmed cell death, either too much or too little, has been shown to cause undesirable micro-environments which can lead to disease development.

Self-defence/self-sacrifice: In addition to its contribution to embryonic development and homeostasis in an organism, programmed cell death also acts as a defense mechanism against viral infection, hostile

environments (eg., growth factor withdrawal) , harmful cellular insults (eg., UV damage) and autoreactive lymphocytes (Reed, 1994). This intrinsic suicide program not only eliminates potentially dangerous cells, but, more importantly, it also preserves the integrity of the organism as a whole.

1.4 Programmed Cell Death and Diseases

As programmed cell death is responsible for various fundamental functions in multicellular organisms, its untimely activation or inactivation has been shown to be crucial for the development of various diseases.

Today, one of the fastest growing areas of research is the role of programmed cell death in cancer development. It is believed that the inability to engage PCD contributes to the accumulation of excess cells and disrupts the homeostasis of the organism. One of the earliest and most profound example of the involvement of apoptosis in cancer development is the role of the *bcl-2* gene in B-cell follicular lymphomas (Cleary et al., 1986). Often in this type of non-Hodgkin's disease, Bcl-2 protein is overexpressed due to the t(14;18) chromosomal translocation. It has been shown that overexpression of Bcl-2 blocks apoptosis mediated by various stimuli in cultured cells. Strikingly, mice which carry the *bcl-2* transgene develop B-cell lymphomas, thus mimicking the human phenotype (McDonnell et al., 1989; McDonnell and Korsmeyer, 1991).

While too little cell death may contribute to tumourgenesis, inappropriate cell death has been associated with neurodegenerative conditions such as Alzheimer's disease (Smale et al., 1995), AIDS (Gougeon et al., 1993), and amyotrophic lateral sclerosis, a.k.a. Lou Gehrig's disease (Yoshiyama et al., 1994). The diseases which have been shown to be related

to the deregulation of programmed cell death (apoptosis) are summarized in Table 1.

2. Cell death in Invertebrates

Since the ability to manipulate the execution phase of apoptotic PCD embodies therapeutic potential, major effort has been made in dissecting the signals and components involved. Small invertebrates, like *Caenorhabditis elegans* and *Drosophila melanogaster*, provide powerful yet straight forward models to delineate the constituents of apoptosis because of their simple genetic makeups.

2.1 Caenorhabditis elegans

One of the earlier genetic studies of programmed cell death was performed on the nematode *C. elegans* because of the remarkable invariable series of cell divisions which underlie its development. During this process, 131 of the 1090 somatic cells generated die by apoptosis (Ellis et al., 1991). Genetic mutation analysis has revealed that 14 genes govern the precise progression of this cellular suicide process (Ellis et al., 1991; Steller, 1995).

Three genes, *ced-3*, *ced-4* and *ced-9*, are found to be cardinal for the execution of apoptosis in the nematode. *ced-3* (cell death abnormal/defective) and *ced-4* are essential for the induction of apoptosis. Recessive mutations of these two genes abolish the death of all 131 cells which are destined to die during development (Hengartner, 1996). Lineage study in the neurons of developing *C. elegans* indicated that *ced-4* requires wild type *ced-3* to conduct cell death. In contrast, overexpression of Ced-3 can induce apoptosis in a *ced-4*-independent fashion, showing that *ced-3*

Table 1: Diseases related to Deregulation of Programmed cell death

Diseases associated with inhibition of apoptosis	Diseases associated with increased apoptosis
Cancer	AIDS
Autoimmune disorders	Neurodegenerative disorders
Viral infection	Ischemic injury
	Toxin-induced liver disease
	Myelodyplastic syndromes

functions downstream of *ced-4* (Shaham and Horvitz, 1996). Biochemical studies of these proteins supported this conclusion.

The Mammalian homologues of Ced-3 are the members of the caspase (cysteine aspase) family (Alnemri et al., 1996). The caspases are cysteine proteases which cleave specifically after aspartic acid residues. Like the other caspases, Ced-3 is synthesized as a zymogen containing a prodomain at its amino-terminus. Maturation of Ced-3 involves the cleavage of the pro-enzyme at D220, D374 and D388 (using one letter amino acid code). This produces a functional heterotetramer containing two 17kDa (a.a. #221-374) large subunits and two 13kDa (a.a. #375-503) or 15kDa (a.a. #389-503) small subunits (Xue et al., 1996).

The killing effect of Ced-3 is entirely dependent on its ability to act as a caspase. Mutation of the conserved catalytic cysteine, within the large subunit , to serine (Ced-3mC358S) abolishes Ced-3's ability to autoprocess itself and to cause death *in vivo* (Xue et al., 1996). Consistent with this, the apoptosis-inducing properties of three natural mutant *ced-3* alleles n2427 (G474R), n1129 (A449V) and n2433 (G360S) (Yuan et al., 1993) are proportional to their enzymatic activities (Xue et al., 1996). Although substrates for Ced-3 have not been identified in *C. elegans*, purified Ced-3 exhibits a substrate specificity similar to caspase-3 (CPP32) and prICE, a caspase activity detected in S/M apoptotic cell extracts from chicken cells (Lazebnik YA et al., Nature 371, 346, 1994). *In vitro*, Ced-3 cleaves both Ced-3 and caspase-3 (CPP32) precursors, poly(ADP-ribose) polymerase (PARP) and baculoviral protein p35, but not cytokine response modifier A gene product (Crm A), procaspase-1 (ICE) and pro-interleukin-1β (IL-1β), a substrate of caspase-1 (Xue et al., 1996).

The second cell death gene, *ced-4*, encodes a 63kDa novel protein containing two putative "EF hand" motifs (Yuan and Horvitz, 1992) and a putative "death effector domain" (Bauer et al., 1997). "EF hand" motifs are found in calcium binding proteins (Nakayama and Kretsinger, 1994) while the "death effector domains" are found in the adaptor molecules present in the Fas/TNFR1 death-inducing signaling complexes (Chinnaiyan et al., 1995). However, the biochemical functions of these motifs in Ced-4 are yet to be confirmed. In addition, Ced-4 also contains a consensus sequence for nucleotide-binding P-loop (A/GXXXXGKS/T using the one letter amino acid code) consisting of residues 158-165 (James et al., 1997). Using the ATP analogue 5'-fluorosulphonyl-benzoyladenosine (FSBA), it was illustrated that Ced-4 binds to ATP and deletion of the P-loop ablates this binding activity (Chinnaiyan et al., 1997a).

Overexpression of Ced-4 alone fails to cause apoptosis in both human and insect cells (Ng F et al unpublished; Seshagiri and Miller, 1997; Wu et al., 1997a), albeit some studies argue otherwise (Chinnaiyan et al., 1997b; James et al., 1997). Nevertheless, it is clear that co-expression of Ced-4 promotes Ced-3 activation *in vivo* and potentiates the killing effect of the protease in co-transfected cells (Chinnaiyan et al., 1997a; Seshagiri and Miller, 1997). Consistent with this, expression of catalytically inactive Ced-3 can abrogate Ced-4's ability to activate Ced-3 and thus cell death (Chinnaiyan et al., 1997a; Seshagiri and Miller, 1997). The domains which are critical for the stimulation of Ced-3-dependent killing by Ced-4 have been localized to the Ced-3 prodomain and the nucleotide-binding P-loop of Ced-4. It has been shown that Ced-4 fails to enhance the death effect of a Ced-3 mutant containing L27P (leucine to phenylalanine) mutation in the prodomain and the expression of Ced-4 lacking the P-loop or of the P-loop

mutant Ced-4^{K165R} cripples the processing of Ced-3 (Chinnaiyan et al., 1997a; Seshagiri and Miller, 1997).

While both Ced-3 and Ced-4 trigger programmed cell death, Ced-9 blocks apoptosis and protects the cells from untimely death (Hengartner et al., 1992). Based on genetic studies, the function of *ced-9* is essential during the development of *C. elegans*. Loss-of-function mutation of *ced-9* contributes to embryonic lethality by failing to protect cells which normally survive (Hengartner et al., 1992) . The mammalian homologue of Ced-9 is the protein product of the human proto-oncogene *bcl-2,* which shares 23% sequence identity with Ced-9 (Hengartner and Horvitz, 1994). Expression of Bcl-2 rescues the Ced-9 loss-of-function mutant, indicating that the nematode cells share a common death pathway with mammalian cells.

Transgenic experiments in developing *C. elegans* neurons demonstrated that *ced-9* blocks apoptosis induced by *ced-3* and *ced-4* by acting upstream of these cell death genes (Shaham and Horvitz, 1996). Intriguingly, these studies also revealed that concomitant expression of Ced-4 is required for Ced-9 inhibition of Ced-3-induced cell death, suggesting Ced-9 functions at least by part via Ced-4 (Shaham and Horvitz, 1996). Recently, Ced-4 was shown to interact simultaneously with both Ced-3 and Ced-9 (Chinnaiyan et al., 1997b; Wu et al., 1997b). This suggests that Ced-9 inactivates Ced-3 indirectly through its physical contact with Ced-4 (Seshagiri and Miller, 1997; Wu et al., 1997a,b). It is conceivable that Ced-9 regulates the nucleotide-binding activity of the Ced-4 P-loop, since this domain is required for the processing of Ced-3 (Chinnaiyan et al., 1997a; Seshagiri and Miller, 1997). Moreover, co-expression of Ced-9 relocates Ced-4 to subcellular membrane sites, such as mitochondria and endoplasmic reticulum. This suggests that Ced-9 may function by

sequestering Ced-4 from the cytosol where the activation of Ced-3 occurs (James et al., 1997; Wu et al., 1997b). Of note, however, is the observation that deletion of the carboxy-terminal hydrophobic domain of Ced-9, which is responsible for its subcellular localization, did not ablate its ability to associate with Ced-4 and inhibit Ced-4-mediated activation of Ced-3 in insect cells (Seshagiri and Miller, 1997). This result is in agreement with a parallel study in mammalian cells which demonstrated that deletion of the carboxy-terminal signal anchor sequence of Bcl-2, the Ced-9 mammalian homolog, impaired, but did not eliminate, its protective effect (Nguyen et al., 1994).

2.2 Drosophila Melanogaster

In addition to *C. elegans, Drosophila Melanogaster* (fruit fly) has also been employed for the genetic study of apoptosis. The work done by Steller and colleagues identified two apoptosis-inducing genes, *reaper (rpr)* (named in honour of the Grim Reaper) and *hid* (head involution defective) (Grether et al., 1995; White et al., 1994). The two genes are located at the (75C1,2) locus. Deletion of this region dramatically decreases cell death during development.

rpr encodes a 65 amino acid novel protein while *hid* encodes a 410 amino acid protein both of which lack significant homolgy to other death-inducing proteins identified in mammals and *C. elegans*. Interestingly, however, Rpr shows weak sequence homology to the "death domains". Death domains are protein-protein interaction motifs which are essential to Fas/TNFR1 receptor complexes for their ability to transduce death signals (Baker and Reddy, 1996). Co-expression of baculovirus p35 protein, which has been shown to block apoptosis in *Drosophila*, nematodes and mammals

by inactivating cell death proteases (Hay et al., 1994; Martinou et al., 1995; Rabizadeh et al., 1993; Sugimoto et al., 1994), can suppress both Rpr and Hid-elicited cell death, confirming the direct effect of *rpr* and *hid* in activating apoptosis (Grether et al., 1995; White et al., 1994). Unlike *ced-3* and *ced-4* in *C. elegans*, cell death effects of both *rpr* and *hid* are additive and each gene can activate apoptosis in the absence of the other when overexpressed. Moreover, their activities may reside upstream of the core cell death machinery, since cell death can still occur in the absence of both genes.

A new gene termed "*grim*" has also joined the *Drosophila* cell death gene family. It maps between *rpr* and *hid* at the (75C1,2) locus. The pattern of the onset of *grim* expression correlates with the spatial and temporal pattern of programmed cell death during the fly development (Chen et al., 1996b). Ectopic expression of grim induces apoptosis in transgenic flies, and this is also inhibitable by the baculoviral p35 protein. Since *grim* functions independently of *rpr* and *hid*, it further supports the existence of parallel pathways in *Drosophila* which eventually converge to a common cell death route (Chen et al., 1996b).

While *rpr, hid* and *grim* were identified because of their involvement in development, a fourth cell death gene, *doom*, was isolated due to its interaction with a family of baculovirus inhibitor-of-apoptosis proteins (Iaps) (Harvey et al., 1997). Like the other three cell death genes in *Drosophila*, expression of Doom in insect cells also elicits apoptotic cell death.

Although the relationship between *rpr, hid, grim* and *doom* has yet to be further characterized, apoptosis elicited by these genes requires caspase activity . The first *Drosophila* homologue of *ced-3, dcp-1*, was

identified by screening a Drosophila embryonic library with degenerative oligonucleotides derived from the conserved sequences found in *ced-3* and mammalian caspases (Song et al., 1997). DCP-1 (Drosophila caspase-1) shows enzymatic activity similar to Ced-3 and the mammalian caspases, and its overexpression induces apoptosis both *in vivo* and *in vitro*.

3. Cellular components of Apoptosis in Mammalian Cells

While both *C. elegans* and *Drosophila* are simple systems whereby genetic materials can be manipulated easily, programmed cell death in mammalian cells is far more complex. In the past decade, laboratories worldwide have been trying to identify the key components that regulate apoptosis, the signaling pathways which lead to self-destruction and the factors which discriminate the "suicidal" cells from normal cells in the mammalian system. The common goal behind this "apoptosis frenzy" is not merely academic, but, more importantly of course, it is to exploit the therapeutic potential of the programmed cell death pathway as a target for disease treatment.

3.1 Bcl-2 Family

3.1.1 Bcl-2

The research of apoptotic programmed cell death literally exploded following the discovery of the human proto-oncogene *bcl-2* (B-cell lymphoma/leukemia 2). It was identified at the t(14;18) chromosomal translocation which is commonly detected in B-cell follicular lymphomas (Pegoraro et al., 1984; Tsujimoto et al., 1985; Tsujimoto et al., 1984). This in-frame translocation places the *bcl-2* gene under the control of an immunoglobulin heavy-chain (IgH) promoter, leading to the

overexpression of both *bcl-2* mRNA and Bcl-2 protein (Cleary et al., 1986). The evidence which firmly suggested that abnormal upregulation of Bcl-2 played an active role in tumourgenesis was obtained from transgenic animal models. Mice carrying a *bcl-2*-immunoglobulin minigene which structurally mimicked the t(14;18) showed pathology that was highly reminiscent of the human B-cell lymphomas (McDonnell et al., 1989; McDonnell and Korsmeyer, 1991).

The human *bcl-2* gene encodes a 26kda protein which lacks significant sequence homology with previously described proteins. However, it does share a low degree of sequence similarity (22% identity; 42% similarity) with an Epstein-Barr virus protein, BHRF-1 (Cleary et al., 1986). Northern analysis shows that Bcl-2 is expressed within various organized tissues, including germinal center, breast, thyroid, prostate, pancreas, nervous system, gastrointestinal system, skin, bone marrow and the thymus. The spatial distribution of Bcl-2 demonstrates that it is topographically restricted to most, but not all, long-lived cell types (Hockenbery et al., 1991).

Initial biological functional studies of Bcl-2 showed that ectopic expression of this protein in an IL-3-dependent pre-B cell line prolonged cell survival upon growth factor withdrawal and that it cooperated with c-myc in transforming the same cells (Vaux et al., 1988). These observations were also extended to other cell types, such as the T-cell Jurkat leukemic line. Although Bcl-2 is capable of cooperating with c-myc to promote cell proliferation, it does not sustain cell growth and transformation on its own (Vaux et al., 1988). Rather, the oncogenic potential of Bcl-2 is conferred by its ability to block apoptosis. Overexpression of Bcl-2 in varies B-cell lines can block all the apoptotic features induced by IL-3 deprivation and promote

cell survival (Hockenbery et al., 1990; Williams et al., 1990; Wyllie et al., 1984). In addition to growth factor withdrawal, Bcl-2 potently suppresses apoptosis elicited by most, but not all, apoptotic stimuli in various cell types ranging from lymphocytes to neuronal cells (Garcia et al., 1992; Kane et al., 1993). Apoptotic stimuli which are inhibitable by Bcl-2 include gamma- and UV-irradiation, chemotherapeutic drugs, viral infection, and p53 expression (Reed, 1994). It broad-range effects imply that Bcl-2 acts on a common cell death pathway to which all these different apoptotic stimuli converge.

As mentioned above, Bcl-2 is involved in an evolutionarily conserved programmed cell death pathway because it can functionally replace Ced-9 in *C. elegans* (Hengartner and Horvitz, 1994). Nevertheless, unlike Ced-9, which is essential for the development of the nematode (Hengartner et al., 1992), Bcl-2 is not required for mammalian development. *bcl-2*$^{-/-}$ mice complete development, although they suffer from polycystic kidney disease, fulminant apoptosis of the thymus and spleen and subsequently early mortality (Veis et al., 1993). This demonstrates that expression of Bcl-2 is not required during embryogenesis, probably due to genetic redundancy. However, it serves as a postnatal survival factor .

Although Bcl-2 does not possess any known structural motifs, it contains a hydrophobic region located at its extreme carboxy-terminus. This hydrophobic tail serves as a membrane anchor signal which targets Bcl-2 to intracellular membrane organelles. Bcl-2 is localized to the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope, with the bulk of the protein facing the cytosol (Akao et al., 1994; Chen-Levy et al., 1989; Monaghan et al., 1992; Nguyen et al., 1993). The correct localization and orientation, but not the particular hydrophobic

signal anchor sequence, of the protein are required for its anti-apoptotic function. Deletion of the signal anchor sequence impairs Bcl-2's ability to protect cells from apoptosis (Alnemri et al., 1992; Nguyen et al., 1994; Tanaka et al., 1993). Interestingly, replacement of this signal anchor sequence of Bcl-2 by the one found in yeast outer mitochondrial membrane protein Mas70 restores its function by targeting the hybrid proteins to mitochondria in a correct orientation (Nguyen et al., 1994).

To date, Bcl-2 is the most characterized anti-apoptotic cellular factor in mammalian cells. However, the molecular mechanism of Bcl-2 is thus far elusive. It has been proposed that Bcl-2 protects against cell death by acting as an anti-oxidant (Hockenbery et al., 1993; Kane et al., 1993). Several studies have indicated that Bcl-2 blocked apoptosis by suppressing the generation of reactive oxygen species (ROS). ROS, which can be induced by apoptotic signals like IL-3 deprivation and glutathione depletion (Hockenbery et al., 1993; Kane et al., 1993), can lead to lipid peroxidation disrupting the cellular network . However, the relevance of Bcl-2's role as an anti-oxidant to its anti-apoptotic activity remains inconclusive. It has been shown that Bcl-2 can protect cells against apoptosis under anaerobic conditions, arguing that the downregulation of ROS production observed in the previous studies is secondary to Bcl-2-mediated protection (Jacobson and Raff, 1995; Shimizu et al., 1995).

Bcl-2 is also proposed to suppress apoptosis by regulating calcium ion fluxes. It has been shown that artificial increase of intracellular Ca^{2+} levels upon chemical treatment can trigger apoptosis. Expression of Bcl-2 can protect against cell death under this condition by inhibiting the Ca^{2+} efflux from the ER (Lam et al., 1994). Although the role of intracellular Ca^{2+} with respect to cell death has been unclear (Lam et al., 1994; Reynolds

and Eastman, 1996), a recent study supported the importance of intracellular Ca^{2+} during apoptosis. It showed that cells which lack the type 1 inositol-1,4,5-trisphosphate receptor (IP_3R-1), a calcium release channel on the endoplasmic reticulum (ER) membrane, were resistant to apoptosis induced by various stimuli due to the inability of cells to increase intracellular calcium ions (Jayaraman and Marks, 1997). Although there is a lack of evidence showing interaction between Bcl-2, which is also found in the ER, and IP_3R-1, it is possible that their activities with respect to the regulation of calcium ions during apoptosis are connected indirectly by other, yet-to-be identified, components.

3.1.2 Bcl-2 family members

Several proteins with sequence homology to Bcl-2 have been isolated by various methods: co-immunoprecipitation, yeast two-hybrid, interactive cloning and screening of cDNA libraries using degenerative primers based on the *bcl-2* sequence. Bcl-2 along with its related proteins constitute a new family which has become central to the study of programmed cell death. Based on their biological functions, these proteins are subcategorized into the anti-apoptotic and proapoptotic subfamilies.

Anti-apoptosis: Bcl-X_L and Bcl-w belong to the anti-apoptotic subfamily led by Bcl-2 (Boise et al., 1993; Gibson et al., 1996). Like Bcl-2, ectopic expression of any of these proteins promotes cell survival. In particular, Bcl-X_L, which displays wider tissue distribution than Bcl-2 (Krajewski et al., 1994), is emerging as another critical player in suppressing apoptosis. Its expression has also been shown to associate with the development of various tumours (Foreman et al., 1996; Krajewska

et al., 1996; Schlaifer et al., 1996) , and with a multidrug resistance phenotype (Minn et al., 1995; Schlaifer et al., 1996).

Despite the fact that Bcl-2 and Bcl-X_L can act similarly in most systems (Shimizu et al., 1996; Shimizu et al., 1995), there are clear functionally differences between the two proteins. For instance, ectopic expression of Bcl-X_L prevents apoptosis induced by immunosuppressant and protein synthesis inhibitors in a murine B-cell line, WEHI-23, while Bcl-2 fails to do so (Gottschalk et al., 1994). They also provide different degrees of protection against Fas-induced apoptosis (Armstrong et al., 1996; Boise and Thompson, 1997; Itoh et al., 1993; Strasser et al., 1995). More striking evidence came from the transgenic knockout animal model. Homologous disruption of the *bcl-X* gene in mice leads to embryonic lethality. The animals die around embryonic day 13 due to excessive cell death in immature neurons in the nervous system and the shortening of the life-span of immature lymphocytes (Motoyama et al., 1995). Bcl-2-deficient mice, on the other hand, complete embryonic development in spite of the fact that they suffer from early mortality (Veis et al., 1993). It shows that Bcl-2 and Bcl-X_L are not functionally equivalent during development, which could simply be due to the fact that Bcl-X_L is much more widely distributed.

Proapoptosis: Despite the fact that the members of the proapoptotic subfamily share sequence homology with Bcl-2/Bcl-X_L proteins, they function to antagonize the protective effects of Bcl-2/Bcl-X_L and promote cell death. These proteins include Bax, Bak, Bik, Bid and Bad (Boyd et al., 1995; Chittenden et al., 1995b; Farrow et al., 1995; Kiefer et al., 1995; Oltvai et al., 1993; Wang et al., 1996; Yang et al., 1995).

The most studied member of this subfamily to date is Bax (Bcl-2-associated X protein). When overexpressed, Bax triggers apoptosis in both mammalian and yeast cells. This can be nullified by co-expression of the Bcl-2/Bcl-X_L proteins (Jurgensmeier et al., 1997). Currently, the proposed mechanism by which Bcl-2 regulates Bax, or vice versa, involves the establishment of a see-saw system between the two counter-acting proteins *in vivo*. In this model, the ratio of the two proteins, the apoptotic agonist (Bax) and antagonist (Bcl-2), determines the fate of the cells. When Bcl-2 is in excess, it favors cell survival. On the contrary, the cells are poised to death if Bax becomes the dominant species (Yang and Korsmeyer, 1996).

Unlike other apoptotic stimuli, Bax-induced cell death has been shown to be mediated by both caspase-dependent and -independent pathways. Expression of caspase inhibitor baculovirus p35 protein and the use of synthetic caspase inhibitors can rescue Bax-induced apoptosis in mammalian cells, but not in yeast cells indicating the downstream effectors are different in these cells (Jurgensmeier et al., 1997). Nevertheless, one can not eliminate the fact that these caspase inhibitors may not inactivate all the existing caspases, both known and unknown.

Recently, *bax* is proposed to be a tumour suppressor gene. Although transgenic mice lacking *bax* developed both hyperplasia and hypoplasia conditions depending on the cellular context (Knudson et al., 1995), *bax*-deficient primary fibroblasts displayed resistance to chemotherapy and p53-induced apoptosis (McCurrach et al., 1997). Moreover, a recent report showed that in the absence of Bax, the ability for p53 to induce apoptosis in tumour cells decreased dramatically (Yin et al., 1997).

3.1.3 The BH (Bcl-2 homology) domains

It has been hypothesized that the two Bcl-2 subfamilies regulate one another's activity by forming homo- and heterodimers. This competitive dimerization between the death agonists (Bax, Bid, Bak and Bad) and antagonists (Bcl-2, Bcl-X_L and Bcl-w) establishes a dynamic equilibrium which determines cell survival or death (Yang and Korsmeyer, 1996). The ability of these proteins to homo- and heterodimerize relies on the presence of conserved Bcl-2 homology (BH) domains. Based on structural and functional analysis, four BH domains have been identified within the Bcl-2 family. Both Bcl-2 and Bcl-X_L contain all four domains while Bax, Bak and Bad contain only the BH1, BH2 and BH3 domains. Bid, a more distant relative of Bcl-2, possesses only the BH3 domain (Kroemer, 1997).

Mutagenesis analysis revealed that Bcl-2/Bcl-X_L homodimerizes through the BH1 and BH2 domains. These two domains are also required for Bcl-2/Bcl-X_L to heterodimerize with Bax and to suppress cell death (Yin et al., 1994). In contrast, BH3 domains found in the proapoptotic molecules are required for both homo- and heterodimerizaion. Bax, which also harbours BH1 and BH2 domains, depends on its BH3 domain to heterodimerize with Bcl-2 (Hunter and Parslow, 1996; Zha et al., 1996). This is also true for Bad and Bak in binding to Bcl-X_L (Chittenden et al., 1995a; Zha et al., 1997). The NMR structure of the Bcl-X_L/Bak peptide complex further substantiates these observations by showing that the Bak peptide, which contained only the BH3 domain of Bak, binds to the hydrophobic cleft formed by the BH1, BH2, and BH3 domains of Bcl-X_L (Sattler et al., 1997).

A growing body of evidence indicates that the cytotoxic effects of the apoptosis agonists are also dependent on the BH3 domains. This was

demonstrated by deletion mapping of Bax, Bad and Bak (Chittenden et al., 1995a; Hunter and Parslow, 1996; Zha et al., 1996; Zha et al., 1997). Moreover, replacement of the BH3 domain of Bcl-2 by the Bax BH3 domain converts Bcl-2 into a death-inducing protein (Hunter and Parslow, 1996).

Unlike the other BH domains, BH4 domains are well conserved among the Bcl-2-like proteins, but not among the proapoptotic family members. Although BH4 domains are dispensable for dimerization, they are required for interactions with proteins outside the Bcl-2 family (e.g. calcineurin and Ced-4) (Chinnaiyan et al., 1997; Shibasaki et al., 1997). Furthermore, deletion of the BH4 domain in Bcl-2 abrogates its anti-apoptotic effect (Hunter et al., 1996). This raises the possibility that the anti-apoptotic properties of Bcl-2-like proteins are not only conferred by their ability to antagonize proapoptotic family members, but also by interaction with other cellular proteins. This hypothesis is supported by the fact that there is functional independency among the Bcl-2-related proteins. Mutations which abolish the interaction between Bcl-X_L and Bax have minimal effects on Bcl-X_L's ability to protect against cell death (Cheng et al., 1996). In addition, mice generated by crossing *bcl-2*$^{-/-}$ and *bax*$^{-/-}$ mice suggest that Bcl-2-repression or Bax-induction of apoptosis can occur independently (Knudson and Korsmeyer, 1997).

3.1.4 Crystal structure of Bcl-XL

As the old saying "a picture is worth a thousand words" states, the X-ray crystallographic structure of Bcl-X_L monomer provides the first three dimensional portrait of the Bcl-2 family (Muchmore et al., 1996). Bcl-X_L consists of seven alpha-helices with a flexible loop domain connecting helices 1 and 2. The structure of Bcl-X_L was found to be analogous to

bacterial toxins namely diphtheria and colicins, whose translocation domains are proposed to form a pH-dependent membrane pore upon dimerization (London, 1992; Parker and Pattus, 1993).

Recently, *in vitro* studies revealed that Bcl-X_L, Bcl-2 and Bax can form channels in synthetic lipid membranes whose properties are pH-dependent (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). Interestingly the presence of Bcl-2 inhibits the activity of the Bax channel, suggesting Bcl-2 may regulate the proapoptotic effects of Bax by interfering with its channel activity (Antonsson et al., 1997). As discussed below, apoptosis triggers the formation of permeability transition pores and subsequently the lost of transmembrane potential of the mitochondria (Hennet et al., 1993; Marchetti et al., 1996). Therefore, it is possible that Bcl-2 family members may form channels/pores in the cytoplasmic membranes where they are located, and regulate the permeability of these membranes directly or indirectly during apoptosis. While this theory is attractive, the channel/pore-forming properties of these proteins *in vivo* has yet to be established.

3.1.5 Bcl-2, Mitochondria and Cell Death

There is a general belief that the mitochondrion is the most important location for Bcl-2 and that the integrity of mitochondria is the "holy grail" that Bcl-2 protects.

One of the observations which supports this hypothesis is that upon induction of apoptosis, cytochrome c is translocated into the cytosol from mitochondria and triggers the activation of caspase-3 (Liu et al., 1996). The presence of Bcl-2 in the outer mitochondrial membrane prevents the release of cytochrome c and blocks cell death (Kluck et al., 1997; Yang et al., 1997).

Interestingly, the protective effect of Bcl-2 can be bypassed by the addition of exogenous cytochrome c *in vitro*, indicating that the release of cytochrome c is a late event of apoptosis (Kluck et al., 1997; Liu et al., 1996).

Moreover, it has been demonstrated that one of the earliest irreversible events of apoptosis is the collapse of the mitochondrial inner membrane potential ($\Delta\Psi_m$) mediated by permeability transition (PT)-pores (Kroemer et al., 1997; Zamzami et al., 1995a,b). Upon the disruption of $\Delta\Psi_m$ an apoptosis-inducing factor (AIF) is released from the mitochondria (Susin et al., 1996). AIF is a pre-formed, 50kDa protein which is proposed to reside in mitochondria. In a cell-free system, it induces nuclear apoptosis which can be blocked by the presence of caspase inhibitors (Susin et al., 1996). Overexpression of Bcl-2 in cells can impede the formation of PT-pores and thus prevent the release of AIF upon the PT-inducing treatments. Whether the formation of the PT-pores is also responsible for release of cytochrome c is debatable, because the release of cytochrome c has been shown to occur with or without the loss of membrane potential (Eskes et la.,1997; Kluck et al., 1997).

How Bcl-2 prevents the loss of $\Delta\Psi_m$ and the formation of PT-pores is not known. However, combined with the *in vitro* observation that Bcl-2 possesses channel/pore-forming property, an intuitive speculation is that Bcl-2 may form channels in the outer mitochondrial membrane in order to stabilize the membrane potential. In addition it may also interact with the constituents of the PT-pores in order to prevent them from opening.

While these data demonstrate the importance of Bcl-2 in the outer mitochondrial membrane, this location may not be exclusive for Bcl-2 function. It may not, in fact, even be necessary in all cases. When Bcl-2 is targeted exclusively to either the mitochondrial or the ER membrane, its

ability to suppress apoptosis becomes cell-type specific (Zhu et al., 1996). The spatial restriction of the anti-apoptotic function of Bcl-2 in various cell types suggests that apoptotic signals may be shuttled between different intracellular organelles to secure a complete collapse of the cellular network. During evolution, therefore, Bcl-2 has been placed at multiple sites to maximized its protection capability.

3.2 The Caspase Family

Human interleukin-1β-converting enzyme (ICE) /caspase-1, which cleaves interleukin-1β (IL-1β) precursor to its mature form (Thornberry et al., 1992), was the first caspase identified to share sequence homology with Ced-3. Sequence alignment revealed that the two proteins share 29% sequence identity (Yuan et al., 1993). Genetic screening revealed that ICE (caspase-1) belongs to a growing family of cysteine proteases called the caspases (cysteine aspase) (Alnemri et al., 1996). These proteases, all of which contain a conserved QACRG pentapeptide sequence harbouring the catalytically active cysteine (Thornberry et al., 1992), cleave carboxy to aspartic acid residues. Using synthetic peptides it was demonstrated that the three residues N-terminal to the aspartic acid residue dictate the substrate specificity of the proteases (Thornberry et al., 1992). Like Ced-3, overexpression of these caspases induces apoptotic cell death.

To date, ten caspases have been identified in mammalian cells (Alnemri et al., 1996). All of them are synthesized as zymogens whose maturation requires cleavage at the internal aspartic acid residues to generate large and small active subunits. This occurs by either autoprocessing or processing by other family members (Takahashi and Earnshaw, 1996). The crystal structures of caspase-1 (ICE) and caspase-3

(Cpp32) revealed that a mature active caspase is a tetramer of two heterodimers formed by the two subunits (Rotonda et al., 1996; Walker et al., 1994).

It is believed that the caspase activity is essential for PCD because expression of cowpox virus cytokine response modifier A (Crm A), a natural caspase inhibitor, or the presence of synthetic caspase inhibitors, can block apoptosis mediated by most stimuli. Moreover, ectopic expression of Bcl-2 prevents the activation of caspase-3 and blocks apoptosis (Boulakia et al., 1996), suggesting that the caspases act in the same cell death pathway as the Bcl-2-related proteins. Since direct interaction between the caspases and Bcl-2/Bcl-X_L has not been reported, it is likely that Bcl-2/Bcl-X_L regulate the caspases by limiting the factors required for their activation. Two of the potential candidates are cytochrome c and AIF (Kluck et al., 1997; Liu et al., 1996; Susin et al., 1996; Yang et al., 1997). As discussed above, the release of these factors from mitochondria can trigger the activation of caspase-3 resulting in cell death. Expression of Bcl-2 or Bcl-X_L prevents the release of these factors and blocks the activation of the protease (Kharbanda et al., 1997; Kluck et al., 1997; Liu et al., 1996; Susin et al., 1996; Yang et al., 1997).

Although it is well accepted that the caspases, especially caspase-3-like proteases, are central in executing the "death sentence" in mammalian cells, recent studies show that broad-spectrum synthetic caspase inhibitors failed to block apoptosis induced by Bax and E4 orf 4 (Lavoie et al., 1998; Xiang et al., 1996). In addition, apoptosis induced by Bax in yeast cells also appears to be caspase-independent (Jurgensmeier et al., 1997). It suggests that the caspase activity may be essential to most, but not all, apoptotic stimuli and that a caspase-independent pathway may

exist (Jurgensmeier et al., 1997; Lavoie et al., 1997; Xiang et al., 1996). Of course, one cannot discard the possibility that such caspase inhibitors may not be effective in blocking other, yet-to-be identified caspases.

In general, the caspases can be subdivided into two main hierarchy groups: the initiator and effector caspases. During apoptosis, the caspase cascade starts as the initiator caspases activate downstream effector caspases, which in turn cleave specific targets to ensue the irreversible cellular destruction. To date, several cellular substrates of caspases have also been identified. These include pro-IL-1β (Thornberry et al., 1992), Poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994), PITSLRE kinase (Beyaert et al., 1997) and U1-70kda small ribonucleoprotein (Tewari et al., 1995a). At this point, it is not clear whether the cleavage of these proteins constitutes the key events of apoptosis or if their cleavage is simply the consequence of cell death, because the biological effects of these cleavage events are unknown.

3.2.1 Caspase-8 (FLICE) : an initiator caspase

In addition to the regions which give rise to the two active subunits, caspases also possess a large amino-terminal prodomain. Although the function of this prodomain has not been investigated extensively, the prodomain of FLICE (FADD-like ICE/Ced-3-like protease) (caspase-8) is responsible for its recruitment to the Fas death-inducing signaling complex (DISC) (Muzio et al., 1996). The prodomain of caspase-8 contains two motifs which are homologous to the "death effector domain" (DED) found in FADD (Fas-associated protein with death domain) (Chinnaiyan et al., 1995). Upon oligomerization of the receptors induced by ligands or antibodies, FADD is recruited to the DISC, which in turn recruits caspase-8 through the DEDs.

The recruitment of caspase-8 to the Fas DISC is the first biochemical evidence which physically links a cell surface death-inducing receptor with a cytoplasmic proapoptotic caspase. It also demonstrates that caspase-8 is an apical caspase in the caspase cascade. Consistent with this, it has been shown that recombinant caspase-8 can directly activate other caspases, such as caspase-3 and caspase-9, *in vitro* (Muzio et al., 1997) . Furthermore, its ability to induce DNA fragmentation in isolated nuclei depends on the presence of cytosolic extracts, indicating the requirement for downstream caspases (Muzio et al., 1997).

In an attempt to delineate the sequence of events which comprise the caspase cascade, a recent study placed Bcl-X_L downstream of caspase-8, but upstream of caspase-7 (Srinivasan et al., 1997). These researchers showed that Bcl-X_L blocked Fas or TNF-induced apoptosis and prevented the processing of caspase-7, but not caspase-8, in the MCF-7 breast carcinoma cell line. Consistent with this, micro-injection of active caspase-8 did not overcome the protective effect of Bcl-X_L. Nevertheless, one must also note that Fas and TNFR1 signalings are restricted to a small set of external stimuli (i.e. ligands or antibodies), the hierarchy presented in this studies may be specific to this system.

3.2.2 Caspase-3 (Cpp32): an effector caspase

Another caspase which has been extensively studied is caspase-3. It is thought to act as an effector caspase, cleaving specific cellular substrates during apoptosis. Several lines of evidence support this hypothesis.

First, caspase-3 shares the highest sequence homology with Ced-3, the only known *C. elegans* caspase. Apoptosis is likely to be simpler in *C. elegans*, given that the decision to die is genetically programmed rather

than a response to external stimuli. Thus, activator caspases should not be required. Second, caspase-3 can be activated by divergent apoptotic stimuli, including adenovirus E1A , irradiation and anti-Fas treatment (Boulakia et al., 1996; Kharbanda et al., 1997; Tewari et al., 1995b). Third, unlike caspase-1, caspase-2, and caspase-4, caspase-3 is capable of cleaving the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) *in vitro* (Nicholson et al., 1995; Tewari et al., 1995b). Moreover, it does so with similar enzymatic kinetics as the "prICE" (protease resembling ICE) activity in cells committed to apoptose by aphidicolin arrest (Lazebnik et al., 1994). PARP cleavage is a common feature in apoptosis, and has therefore been suggested to mark a convergence point of various apoptotic pathways. Fourth, various studies have placed caspase-3 activation downstream of other apoptotic events. *In vitro* reconstitution of apoptosis has demonstrated that the translocation of cytochrome c from mitochondria into the cytosol is required for the activation of caspase-3. The presence of Bcl-2 can inhibit this translocation and block the activation of the protease (Kluck et al., 1997; Liu et al., 1996; Yang et al., 1997). However, once caspase-3 is activated, Bcl-2 is no longer capable of blocking apoptosis (Kluck et al., 1997; Yang et al., 1997);. Moreover, overexpression of Bcl-X_L, which acts downstream of caspase-8 (Srinivasan A et al., CSH meeting on PCD, 1997), also inhibits the release of cytochrome c into the cytosol upon irradiation-induced apoptosis (Kharbanda et al., 1997) and blocks the activation of caspase-3. All of these data are consistent with the hypothesis that caspase-3 acts downstream of the activator caspase(s), such as caspase-8, and that its activation is a distal event.

Mice which lack this protease display decreased apoptosis in the developing brain (Kuida et al., 1996), indicating that it is essential for cell

death in neural cells. However, it does not appear to be required for all cell death: thymocytes of caspase-3-deficient mice are still capable of undergoing apoptosis (Kuida et al., 1996). This implies that a set of effector caspases may exist in mammalian cells. Indeed, Caspase-6 also shows activity towards PARP (Fernandes et al., 1995) and may function in a similar manner to caspase-3.

In conclusion, the caspase "cascade" is a complicated process, and may contain several feedback loops designed to increase the fidelity of the life or death decision. The precise hierarchy may also vary between cell types in order to predispose cells to respond to certain stimuli.

3.3 Mammalian Ced-4-like Molecule(s)

It has been shown that caspase-8 co-precipitates with Bcl-X_L, although direct interaction between the two proteins has not been detected (Chinnaiyan et al., 1997b). A mammalian Ced-4-like cellular factor is postulated to mediate the this association. In *C. elegans* Ced-4 acts as an adaptor molecule which physically links Ced-9 to Ced-3 and indirectly contributes to the regulation of Ced-3 by Ced-9 (Chinnaiyan et al., 1997a,b; Wu et al., 1997b). A similar observation is seen in mammalian cells. When co-expressed in cultured human cells, both Bcl-X_L and Caspase-8 bind to Ced-4 concurrently, just as Ced-9 and Ced-3 do (Chinnaiyan et al., 1997b). A carboxy-terminal truncation mutant of Ced-4 (Ced-4ΔC), however, fails to interact with Bcl-X_L and attenuates the ability of Bcl-X_L to precipitate caspase-8 presumably by acting as a competitive inhibitor of the mammalian counterpart of Ced-4 (Chinnaiyan et al., 1997b).

Furthermore, binding studies of Ced-4 and the caspases indicate that Ced-4 and Ced-4-like molecule(s) preferentially associate with caspases

containing large prodomains, such as caspase-8 and Ced-3, but not with caspase-3, which contains a small prodomain (Chinnaiyan et al., 1997b). This finding seems contradictory to the proposal that caspase-3 is the functional equivalent of Ced-3 in mammalian cells. However, it may simply suggest that the Ced-3-like proteases in higher eukaryotes have evolved to accommodate the need for a higher order of regulation as the complexity of stimuli increases. This hypothesis accounts for the need for a family of caspases in mammalian cells, although their functions may overlap in some areas.

Recently, Wang and colleagues reported the identification of a 130kDa cellular protein, apoptosis protease-activating factor 1 (Apaf-1), as the potential mammalian Ced-4 candidate. Amino acids 2-85 of Apaf-1 show 21% sequence identity to Ced-3 prodomain while the region between residues 86-405, which harbours two consensus nucleotide-binding P-loops, shares 22% sequence identity to Ced-4 (Zou et al., 1997). These studies revealed that Apaf-1 binds to cytochrome c, which is released from the mitochondria upon the induction of apoptosis, and triggers the events that lead to the activation of the caspase-3. Since neither Apaf-1 nor cytochrome c shows interaction with caspase-3, the activation of caspase-3 by the Apaf-1/cytochrome c complex may result from the activation of other upstream caspases. In fact, in *in vitro* apoptosis system using S-100 extracts, caspase-9 is found to complex with Apaf-1, in the presence of cytochrome c and dATP, triggering the activation of caspase-3 and the caspase cascade (Li et al., 1997). The requirement for cytochrome c and dATP maybe is to cause conformation changes allowing the interaction between Apaf-1 and caspase-9 (Li et al., 1997). Although Apaf-1/cytochrome c/caspase-9 complex is functionally analogous to Ced-4-mediated Ced-3 activation in *C.*

elegans, whether it can interact with Bcl-2/Bcl-X_L proteins remains to be determined.

These studies indicate that the basic cell death regulatory paradigm of *C. elegans* is preserved in mammalian cells, however, they are suggestive of a more convoluted system in higher eukaryotes.

4. Viral gene products

As discussed above, programmed cell death functions to defend host cells from viral infection. Different viruses circumvent such cellular surveillance systems by producing viral proteins whose functions mimic the cellular components involved in apoptosis. At first, the intrinsic cellular suicide process must be suppressed by anti-apoptosis viral proteins to allow efficient viral replication. Later, the transfer of the viral progenies can be facilitated by apoptosis, via the phagocytosis of the apoptotic cells by the neighboring healthy cells.

Studies in human adenovirus (Ad) provided the first clear evidence linking virus-induced cytotoxicity to PCD (Ezoe et al., 1981). Two of the early regions of adenovirus type 5 encode both proapoptotic and anti-apoptotic viral proteins. The two splice variants of the E1A gene, E1A 12S and E1A 13S , can induce apoptosis by both p53-dependent and p53-independent mechanisms (Boulakia et al., 1996; Lowe and Ruley, 1993), and stimulate cell proliferation at the same time (Rao et al., 1992; Shenk and Flint, 1991; White et al., 1991). Therefore, manifestation of the transforming properties of the E1A region requires the suppression of its apoptotic activities by co-expression of the E1B gene. The E1B gene encodes two apoptotic suppressors, the 19kDa and 55kDa proteins (Debbas and White, 1993; Rao et al., 1992). E1B-19k shares sequence homology with mammalian Bcl-2 and

is considered to be a Bcl-2 family member (Chiou et al., 1994). Expression of Bcl-2 has been shown to block apoptosis mediated by infection of 19K-deficient Ad mutants (Nguyen et al., 1994; Rao et al., 1992). In addition, by working in synergy with E1A, Bcl-2 can replace E1B 19K during transformation of primary cells (Chiou et al., 1994; Nguyen et al., unpublished).

Both Bcl-2 and E1B-19k interact with a common set of proteins which include Bak (Bcl-2 homologous antagonist/killer) (Chittenden et al., 1995b; Farrow et al., 1995; Kiefer et al., 1995), Bax (Chen et al., 1996a; Han et al., 1996), and three unrelated proteins, Nip1-3, whose function is as yet unknown (Boyd et al., 1994). Thus, Bcl-2 and E1B 19K may use similar biochemical mechanisms to block apoptosis. Consistent with this, the activation of Caspase-3 induced by 19K-deficient Ad mutants is inhibitable by ectopic expression of either Bcl-2 or E1B 19K (Boulakia et al., 1996). Although there are functional similarities between Bcl-2 and E1B-19k, the viral protein displays a different subcellular localization than Bcl-2, suggesting that functional differences may exist between the two proteins (White et al., 1984).

The second protein encoded by the E1B transcription unit is the E1B-55K protein. Unlike E1B-19K, it blocks apoptosis by serving as a transcriptional repressor through its interaction with p53 (Yew et al., 1994). In addition to its role in triggering cell cycle arrest, p53 also induces apoptosis (Howes et al., 1994; Morgenbesser et al., 1994; Symonds et al., 1994). Genetic studies have shown that p53 is the most frequently mutated gene in human tumours. Transgenic mice lacking functional p53 display high rates of tumourgenesis, although they appear normal during development (Donehower et al., 1992). Another viral protein which also

targets p53 is the human papilloma virus (HPV) E6 protein. It binds to a cellular protein termed E6-AP (E6 associated protein) and forms a E6-E6-AP complex which functions as a ubiquitin-protein ligase. The complex interacts with p53 and promotes the degradation of p53 by the ubiquitin-dependent pathway (Scheffner et al., 1993).

As discussed above, the cowpox virus *crm A* gene (cytokine response modifier gene) encodes a protein which blocks apoptosis by inhibiting the caspases. Crm A is a member of the serpin family, which are serine protease inhibitors (Carrell et al., 1987). Initially, Crm A was identified as an inhibitor of Caspase-1 (Ray et al., 1992). Later, it was shown to suppress apoptosis in a wide range of systems, including Fas/TNF mediated apoptosis (Tewari and Dixit, 1995) and growth factor withdrawal (Wang et al., 1994). The mechanism of action of Crm A is competitive inhibition of the caspases (Hengartner and Horvitz, 1994; Tewari and Dixit, 1995; Wang et al., 1994). It contains a caspase cleavage site and can actively compete for the enzyme binding site. Like Crm A, baculovirus p35 protein also possesses a caspase cleavage site and inhibits apoptosis by competing for the caspases. However, unlike Crm A, upon cleavage p35 undergoes conformational changes and forms a stable complex with the active caspase, and thus inactivates the enzyme (Bump et al., 1995; Xue and Horvitz, 1995).

5. Fas/TNFR1 Signaling complexes

Cell surface receptors TNFR1 (TNF receptor 1) and Fas, both of which belong to the TNF cell surface receptor superfamily, mediate apoptotic programmed cell death upon interaction with their ligands, TNF and FasL, respectively. Mutagenesis studies identified a novel protein-

protein interaction motif termed the "death domain" (DD) in the cytoplasmic domains of the two receptors (Itoh and Nagata, 1993; Tartaglia et al., 1993). Removal of the death domains from TNFR1 and Fas inhibits their ability to induce cell death by abolishing the recruitment of downstream effectors to the death-inducing signaling complexes (DISC) (Baker and Reddy, 1996; Kischkel et al., 1995; Tartaglia et al., 1993).

In a yeast two-hybrid screen, FADD (Fas-associating protein with death domain), which also contains a death domain at its carboxy-terminus and a novel "death effector domain" (DED) at its amino-terminus, was isolated as a Fas interacting protein (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD functions as an adaptor molecule for the recruitment of caspase-8 to the Fas DISC. In this complex, FADD associates with the death domain of Fas through death domain to death domain interaction (Boldin et al., 1995; Chinnaiyan et al., 1995). Procaspase-8, which contains two DEDs in its prodomain is in turn recruited to the complex through DED to DED interaction with FADD (Muzio et al., 1996). Presumably, procaspase-8 then undergoes auto-processing and the active caspase-8 is released from the DISC to trigger the caspase cascade. As mentioned, TNFR1 also contains a DD in its cytoplasmic domain. Although FADD does not interact with TNFR1 directly, it can be recruited to TNFR1 through another adaptor molecule termed TRADD (TNF-R1-associated death domain protein) (Hsu et al., 1996; Hsu et al., 1995). This finding suggests that both Fas and TNFR1 employ a common signaling pathway to induce apoptosis through the recruitment of caspase-8.

To date, caspase-8 is the only caspase which is directly associated with the cell surface receptor. Its recruitment to the DISC not only places caspase-8 at the apex of TNFR1/Fas-induced apoptosis, but it also suggests

that a similar recruitment scenario may exist by which initiator caspases can be directed to intracellular sites to initiate the caspase cascade.

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Experimental Objectives:

While extensive studies have shown that Bcl-2 family members are involved in the regulation of apoptosis, the manner in which these proteins regulate apoptosis is poorly understood. For example, inhibition of caspase activity is a potential activity of anti-apoptotic Bcl-2 family members. However, how such a regulation might occur is unclear given that Bcl-2 family members and caspases fail to interact directly. In order to advance our current knowledge on how Bcl-2/Bcl-X_L proteins function *in vivo*, I have undertaken an approach designed to identify other Bcl-2/Bcl-X_L interacting proteins. A complete characterization of such proteins will shed light on the mechanism of action of Bcl-2/Bcl-X_L, and thereby provide a central point in the apoptotic network which may be amenable to therapeutic manipulation.

Chapter II

p28Bap31, a Bcl-2/Bcl-X_L-and Procaspase-8-associated protein in the Endoplasmic Reticulum

p28 Bap31, a Bcl-2/Bcl-X_L- and Procaspase-8–associated Protein in the Endoplasmic Reticulum

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Abstract. We have identified a human Bcl-2–interacting protein, p28 Bap31. It is a 28-kD (p28) polytopic integral protein of the endoplasmic reticulum whose COOH-terminal cytosolic region contains overlapping predicted leucine zipper and weak death effector homology domains, flanked on either side by identical caspase recognition sites. In cotransfected 293T cells, p28 is part of a complex that includes Bcl-2/Bcl-X_L and procaspase-8 (pro-FLICE). Bax, a pro-apoptotic member of the Bcl-2 family, does not associate with the complex; however, it prevents Bcl-2 from doing so. In the absence (but not presence) of elevated Bcl-2 levels, apoptotic signaling by adenovirus E1A oncoproteins promote cleavage of p28 at the two caspase recognition sites. Purified caspase-8 (FLICE/MACH/Mch5) and caspase-1(ICE), but not caspase-3 (CPP32/apopain/Yama), efficiently catalyze this reaction in vitro. The resulting NH_2-terminal p20 fragment induces apoptosis when expressed ectopically in otherwise normal cells. Taken together, the results suggest that p28 Bap31 is part of a complex in the endoplasmic reticulum that mechanically bridges an apoptosis-initiating caspase, like procaspase-8, with the anti-apoptotic regulator Bcl-2 or Bcl-X_L. This raises the possibility that the p28 complex contributes to the regulation of procaspase-8 or a related caspase in response to E1A, dependent on the status of the Bcl-2 setpoint within the complex.

DESPITE the complexity of signals that can induce apoptotic programmed cell death, many appear to converge on a common execution pathway that is initiated upon pro-enzyme activation of the Ced-3/ICE (caspase) family of cysteine proteases (Kumar and Lavin, 1996; Alnemri et al., 1996). There are at least 10 known members of the family whose activities lead to site-specific cleavage and consequent activation/inactivation of various target molecules. Additionally, these enzymes may operate as part of a cascade in which initiator caspases such as caspase-8 (FLICE/MACH/Mch5) and caspase-10 (Mch4) activate downstream effector caspases such as caspase-3 (CPP32/apopain/Yama; Fernandes-Alnemri et al., 1996; Srinivasula et al., 1996; Muzio et al., 1997). Insight into the likely mechanism of activation of initiator caspases has come with the finding that the large pro-regions of initiator caspases contain a death effector domain that physically links these pro-enzymes to an apoptotic signaling complex. In the case of the Fas (CD95/Apo-1)/TNFR-1 complexes, recruitment of procaspase-8 involves the adaptor molecule FADD via interactions between the death effector domains within the two molecules (Boldin et al., 1996; Muzio et al., 1996). Fas and TNFR-1, however, are highly restricted in the death signals to which they respond. An important question, therefore, is the extent to which the Fas/TNFR-1 paradigm extends to other apoptotic signaling pathways.

For many of these other death pathways, the decision to induce apoptosis in response to specific death signals depends on the status of various cellular regulators, including p53 and the Bcl-2Bax family set point (White, 1996; Yang and Korsmeyer, 1996). The latter arises through heterodimerization between the Bcl-2/Bcl-X_L (Hockenbery et al., 1990; Strasser et al., 1991; Boise et al., 1993) and Bax/Bak (Oltvai et al., 1993; Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995) family of suppressors and promoters, respectively, in which the ratio of the heterodimerizing partners determines the outcome (cell death or cell survival) in response to various death signals. Bad, a more distantly related family member, is a regulator of the set point (Yang et al., 1995) by a mechanism that is governed by phosphorylation (Zha et al., 1996). This may involve Bcl-2–dependent recruitment of Raf-1 kinase (Wang et al., 1996*a*), albeit indirectly.

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Although it is now known that Bcl-2/Bcl-X_L controls the apoptotic execution pathway at a point that is either at or upstream of pro-enzyme activation of CPP32 (Armstrong et al., 1996; Boulakia et al., 1996; Chinnaiyan et al., 1996), how this is achieved remains to be elucidated. It is undoubtedly relevant, however, that Bcl-2/Bcl-X_L family members are integral membrane proteins with a restricted subcellular distribution. They are anchored in the mitochondrial outer membrane and ER/nuclear envelope via a single COOH-terminal transmembrane segment, leaving a protease-sensitive domain exposed to the cytosol (Krajewski et al., 1993; Nguyen et al., 1993; Gonzalez-Garcia et al., 1994). Recent studies have revealed intriguing pore-forming properties for the cytosolic domain of Bcl-X_L in synthetic lipid bilayers (Minn et al., 1997), raising the possibility that the cytosolic domain may have the potential to influence channel activities in these organelles. Mitochondria and ER, either in parallel or in cooperation, are well documented to control a number of electrochemical events that may be linked to the induction of apoptosis and to come under the control of Bcl-2 (Hockenbery et al., 1993; Gottlieb et al., 1996; Lam et al., 1994). Moreover, mitochondria may export pro-apoptotic molecules by a Bcl-2–regulated mechanism (Kluck et al., 1997; Kroemer et al., 1997; Yang et al., 1997); one of these, cytochrome c, activates the caspase-3 zymogen when added to cytosolic extracts. Whether or not mitochondria and ER contribute to proximal events during the initiation of the caspase cascade, however, remains to be elucidated. If so, Bcl-2/Bcl-X_L may be strategically positioned within the cell to influence both upstream and downstream events in this pathway, perhaps involving coordinated signaling between the two organelles.

In addition to dimerizing members of the Bcl-2 family itself, several proteins have been suggested as candidate targets for Bcl-2 interaction (Fernandez-Sarabia and Bischoff, 1993; Boyd et al., 1994; Wang et al., 1994; Naumovski and Cleary, 1996). With the exception of Raf-1 and its Bcl-2–interacting effector, Bag-1 (Wang et al., 1996*a*,*b*), however, the majority of these have not been linked functionally to apoptosis. Here, we describe a polytopic integral membrane protein of the ER, p28 Bap31, that is part of a complex that contains Bcl-2 proteins and procaspase-8. In the absence of Bcl-2, p28 itself becomes a target of a FLICE/ICE-related caspase upon induction of apoptosis, resulting in removal of a cytosolic segment that contains overlapping death effector and leucine zipper homology domains. The resulting product, p20, induces apoptosis upon ectopic expression in transfected cells. It may be that p28 is a Bcl-2–regulated component of an apoptosis signaling pathway, possibly involving coordinated ER–mitochondrial events.

Materials and Methods

Cells and Viruses

Human KB cells expressing the neomycin resistance gene (*neo*) either alone or together with *BCL-2* (Nguyen et al., 1994) were cultured in α-MEM supplemented with 10% FBS and 100 U/ml streptomycin and penicillin. After reaching 80% confluency, the medium was replaced with fresh medium containing either no virus or 25–35 PFU/cell adenovirus type 5 lacking expression of E1B 19K (*pm*1716/2072; McLorie et al., 1991) or adenovirus type 5 expressing only the 243R form (12S) of E1A and no E1B products (*dl*520E1B$^-$; Shepherd et al., 1993). After incubation for 1 h at 37°C, fresh medium was added and cells were collected at various times for analysis. Both forms of the virus elicit a cytotoxic response in infected cells that exhibit all of the hallmark features of apoptosis (Nguyen et al., 1994; Teodoro et al., 1995).

Bacterial Expression and Purification of ^{32}P-labeled Bcl-2 Cytosolic Domain for Ligang Blot (Far Western) Analyses

cDNA encoding the cytosolic domain of human Bcl-2 (i.e., lacking the COOH-terminal 21 amino acids) was inserted into the pTrchis vector (Invitrogen, Carlsbad, CA), and standard PCR methodology was used to extend hexahistidine at the COOH terminus to include a heart muscle kinase recognition sequence using the oligonucleotides 5′-CTAGCGCCCGCCGCGCCTCTGTGGAATTCTGAA and 5′-AGCTTTCAGAATTCCACAGAGGCGCGGCGGGCG-3′. The final construct encoded Bcl-2 (amino acids 1–218), hexahis, Arg, Arg, Ala, Ser-COOH, and the protein designated Bcl-2Δc21/his6/HMK. The Bcl-2 portion contained three additional mutations that were introduced for reasons not related to this project (Met 16 to Leu; Lys 17 to Arg; Lys 22 to Arg). Stable epithelial cell lines that express full length Bcl-2 harboring these mutations were found to be as effective as cells expressing wild-type Bcl-2 in countering apoptotic death stimuli (not shown).

Escherichia coli MC1061 was transformed with p*Bcl-2Δc21/his6/HMK*. When 500-ml cultures reached an A_{600} of 0.6, they were treated with 1.0 mM IPTG, and cells were recovered by centrifugation 4 h later. Packed cells (2.5–3.0 ml) were suspended in 15 ml extraction medium (20 mM Na phosphate, pH 7.4, 0.5 M NaCl, 0.05% vol/vol Triton X-100, 10 mM β-mercaptoethanol, 1.0 mM PMSF, and 1.0 mM benzamidine) and were sonicated eight times with a Vibra Cell probe sonicator (Sonics & Materials, Inc., Danbury, CT) operating at setting 7.5 for 15 s at 4°C. The sonicate was adjusted to 10% (vol/vol) glycerol and centrifuged at 25,000 rpm for 30 min at 4°C in a Ti 50.2 rotor (Beckman Instruments, Inc., Fullerton, CA). The supernatant was added to 1.2 ml Ni^{2+}-NTA agarose (QIAGEN Inc., Chatsworth, CA; a 1:1 [vol/vol] mixture with extraction medium) and incubated for 1.5 h at 4°C. The beads were washed extensively in extraction medium containing 20% (vol/vol) glycerol and 22 mM imidazole, and Bcl-2Δc21/his6/HMK was eluted in extraction medium containing 20% glycerol and 0.3M imidazole. 1 liter of induced culture yielded 0.8–1.0 mg protein that was >95% pure. The purified protein was labeled with ^{32}P after incubation with heart muscle kinase (HMK)[1] and ^{32}P[γ-ATP], yielding 2.0–2.5 × 10^6 cpm/μg protein, and it was used for ligand blotting as described in Blanar and Rutter (1992).

Purification and Identification of p20 Fragment

KB cells were cultured in 20 15-cm plates until 80% confluency was reached, and infected with 25 pfu/cell adenovirus type 5 lacking expression of E1B 19K (*pm*1716/2072). After 60 h, total cells (65–70% nonviable, as judged by exclusion of trypan blue) were collected and rinsed, and the packed cells (~1.5 ml) were suspended in 6 ml ice-cold lysis medium containing 10 mM Tris HCl, pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Triton X-100, and 1 mM PMSF) and separated into three equal portions. Each was subjected to sonication for 4 X 10 s using an Artek probe sonicator (Artek Systems Corp., Farmingdale, NY) operating at setting 6.0. The combined sonicates were centrifuged at 11,000 *g* for 20 min, and the supernatant was mixed with 0.25 vol of 5× SDS sample buffer (250 mM Tris HCl, pH 6.8, 50% glycerol, 0.5% bromophenol blue, 10% SDS, and 1 M DTT). The total volume was subjected to preparative 14% SDS-PAGE using a Prep Cell 491 system (Bio Rad Laboratories, Hercules, CA) fitted with a 37-mm diam resolving gel chamber. Fractions were collected at a flow rate of 1 ml/min, assayed for the presence of p20 by ligand blotting using ^{32}P-Bcl-2Δc21/his6/HMK as a probe, and the reactive peak fractions combined and concentrated fivefold in a Centriprep-10 concentrator (Amicon, Inc., Beverly, MA). The concentrated sample was mixed with an equal volume of 0.12% trifluoroacetic acid and subjected to reverse-phase HPLC in a system (1090; Hewlett-Packard Co., Palo Alto, CA) outfitted with a Vydac C4 column (The Nest Group, Inc., Southboro, MA) (0.21 × 20 cm) prefixed with two SDS removal cartridges (2.1 × 20 mm).

1. *Abbreviations used in this paper*: GST, glutathione S-transferase; HMK, heart muscle kinase; TM, transmembrane.

The column was developed with a linear gradient of 0 to 80% *n*-propyl alcohol containing 0.12% trifluoroacetic acid at a flow rate of 0.1 ml/min and was monitored at A_{280}. Fractions (0.1-ml) were collected, and those containing Bcl-2–reactive p20, as judged by ligand blotting, were individually subjected to NH_2-terminal peptide sequence analysis at Harvard Microchem (Harvard University, Cambridge, MA).

Cloning of p28 Bap31/CDM cDNA

The coding region of p28 was cloned by reverse transcription PCR using human fibroblast RNA together with primers derived from the sequence of human *BAP31* (these sequence data are available from GenBank/EMBL/DDBJ under accession number X81817). Conditions were exactly as described by Goping et al. (1995*a*), and we used 5'-TCTCTAGAACAAACAGAAGTACTGGA-3' as the antisense primer, and 5'-GATCTAGACATCTTCCTGTGGGAA-3' as the sense primer. Authenticity was confirmed by DNA sequence analysis.

Glutathione S-Transferase Fusion Proteins

PCR was used to generate cDNA fragments corresponding to p28 amino acids 1–246 (full length), 1–164, 122–164, and 165–246, using primers that contained either 5'-BamH1 or 3'-EcoR1 overhangs, respectively. The primers were 5'-GCGGATCCATGAGTCTGCAGTGGACT-3' and 5'-GCGAATTCTTACTCTTCCTTCTTGTC-3' for p28 amino acids 1–246; 5'-GCGGATCCATGAGTCTGCAGTGGACT-3' and 5'-GCGAATTCAGTCAACAGCAGCTCCCTT-3' for p28 amino acids 1–164; 5'-GCGCGGATCCCTCATTTCGCAGCAGGCC-3' and 5'-GCGAATTCAGTCAACAGCAGCTCCCTT-3' for p28 amino acids 122–164; and 5'-GCGGATCCGGAGGCAAGTTGGATGTC-3' and 5'-GCGAATTCTTACTCTTCCTTCTTGTC-3' for p28 amino acids 165–246. Fragments generated by PCR were digested with BamH1 and EcoR1, inserted between the BamH1 and EcoR1 sites of pGEX-2t (Pharmacia Fine Chemicals, Piscataway, NJ), and the recombinant plasmids introduced into *E. coli* MC1061. Packed cells from 500 ml of induced culture were recovered, suspended in 25 ml PBS and 0.1% Triton X-100, and sonicated using a Vibra Cell probe sonicator operating at setting 7.5 for 4 × 15 s at 4°C. After centrifugation at 25,000 rpm in a Beckman Ti 50.2 rotor for 25 min, the supernatant was recovered, mixed with 750 μl of a 1:1 suspension of glutathione-Sepharose 4B beads, and the mixture rotated at 4°C for 45 min. After extensive washing of the beads in PBS and 0.1% Triton X-100, glutathione S-transferase (GST) fusion protein was eluted with 3 ml of 50 mM Tris HCl, pH 8.0, and 12 mM reduced glutathione.

Antibodies

GST fusion proteins were injected into chickens, and the resulting IgY antibodies were recovered from the eggs, exactly as described by Goping et al. (1995*a*). After adsorption of IgY that reacted with immobilized GST, antibodies specific for p28 sequences were purified by affinity binding to immobilized GST-p28(165-246) or GST-p28(122-164) fusion protein, using the methods described in the Amino Link Plus kit (Pierce Chemical Co., Rockford, IL).

Transient Transfections

CHO LR73 cells were seeded at a density of 5×10^5 cells/well in six-well plates. 24 h later, the cells in each well were transfected by calcium phosphate precipitation with 0.5 μg luciferase reporter plasmid, 10 μg RcRSV-p28 or RcRSV-p20, and 10 μg sheared salmon sperm DNA (Goping et al., 1995*b*). After 24 h, cells were shocked with 15% glycerol and collected 24 h later. Cells from each well were lysed in 0.4 ml 0.5% NP-40 and 50 mM Tris HCl, pH 7.8, and aliquots were assayed for luciferase activity as described previously (Goping et al., 1995*b*). 293T cells in 10-cm culture plates were similarly transfected with 15 μg total plasmid DNA when cells reached 50–60% confluency.

Coimmunoprecipitations

Approximately 30 h after transfection, 293T cells were washed in PBS and homogenized in 1.0 ml lysis medium/10-cm culture plate (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetate, 0.5% vol/vol NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). After centrifugation at 11,000 *g*, the supernatant was incubated with 50 μl of a 1:1 slurry of protein G Sepharose for 1 h at 4°C. The Sepharose was removed and the supernatant incubated with mouse M2 anti-Flag antibody (IBI-A Kodak Co., New Haven, CT) at 4°C for 6 to 8 h, at which time 20 μl of a 1:1 slurry of protein G Sepharose was added. After 1 h 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 9E1D antibody or mouse anti-HA 12CA5 antibody (both from Babco, Berkeley, CA) or rabbit anti-Bax sc-526 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Results

Appearance and Identification of a Bcl-2–interacting Polypeptide after Induction of Apoptosis by E1A

To detect potential Bcl-2–interacting polypeptides by ligand blot (Far Western) analysis, a ^{32}P-labeled probe was constructed by expressing a modified version of the cytosolic domain of Bcl-2 in *E. coli*. To that end, the last 21 amino acids of Bcl-2 were deleted and substituted with hexahistidine (his6) plus an HMK recognition peptide to facilitate purification and ^{32}P labeling, respectively. Isolation conditions were developed in which the recombinant protein was purified as a soluble product comprised of mixed monomers and dimers at pH 7.4, as judged by FPLC molecular sieve chromatography. The ^{32}P-labeled probe (^{32}P-Bcl-2Δc21/his6/HMK) readily detected either recombinant Bcl-2 or Bax as the only radioactive products on a ligand blot of total bacterial lysate (not shown). When used as a probe to analyze potential Bcl-2–interacting polypeptides in cells induced to undergo apoptosis in response to various stimuli (including adenovirus E1A expression or treatment with puromycin), a product of ~20 kD in size (p20) as judged by SDS-PAGE was observed consistently.

Fig. 1 shows one such example after infection of *neo*- and *BCL-2*–expressing human KB cells with adenovirus type 5 producing either 12S E1A mRNA (which encodes 243R E1A protein) or both 12S and 13S E1A mRNAs (which encode 243R and 289R E1A proteins, respectively), but lacking expression of the dominant suppressor of apoptotic cell death, E1B 19-kD protein (19K). Induction of apoptotic cell death by either virus (Nguyen et al., 1994; Teodoro et al., 1995) was accompanied by the appearance of p20 Bcl-2–binding activity, whereas apparent binding to Bax did not change significantly during the time course of infection. Of note, however, is the observation that stable expression of Bcl-2 in these cells countered cell death and prevented the appearance of p20 Bcl-2–binding activity after viral infection.

Identification of the p20 Bcl-2–binding polypeptide was obtained by NH_2-terminal peptide sequence analysis of p20 after its purification by a combination of differential solubilization in detergent, preparative SDS-PAGE, and reverse-phase HPLC (Fig. 2, *A* and *B*). Several individual HPLC fractions were subjected to peptide sequence analysis to detect a polypeptide sequence whose appearance correlated with the appearance of p20 Bcl-2–binding activity (Fig. 2). One candidate sequence emerged, and it was the only sequence that was detected in the peak fraction of Bcl-2–binding activity (Fig. 2 *B*, fraction 54). It showed a perfect match with amino acids 2–11 of human Bap31 (these sequence data available from GenBank/EMBL/DDBJ under accession number X81817)/CDM (these sequence data available from GenBank/EMBL/DDBJ under accession

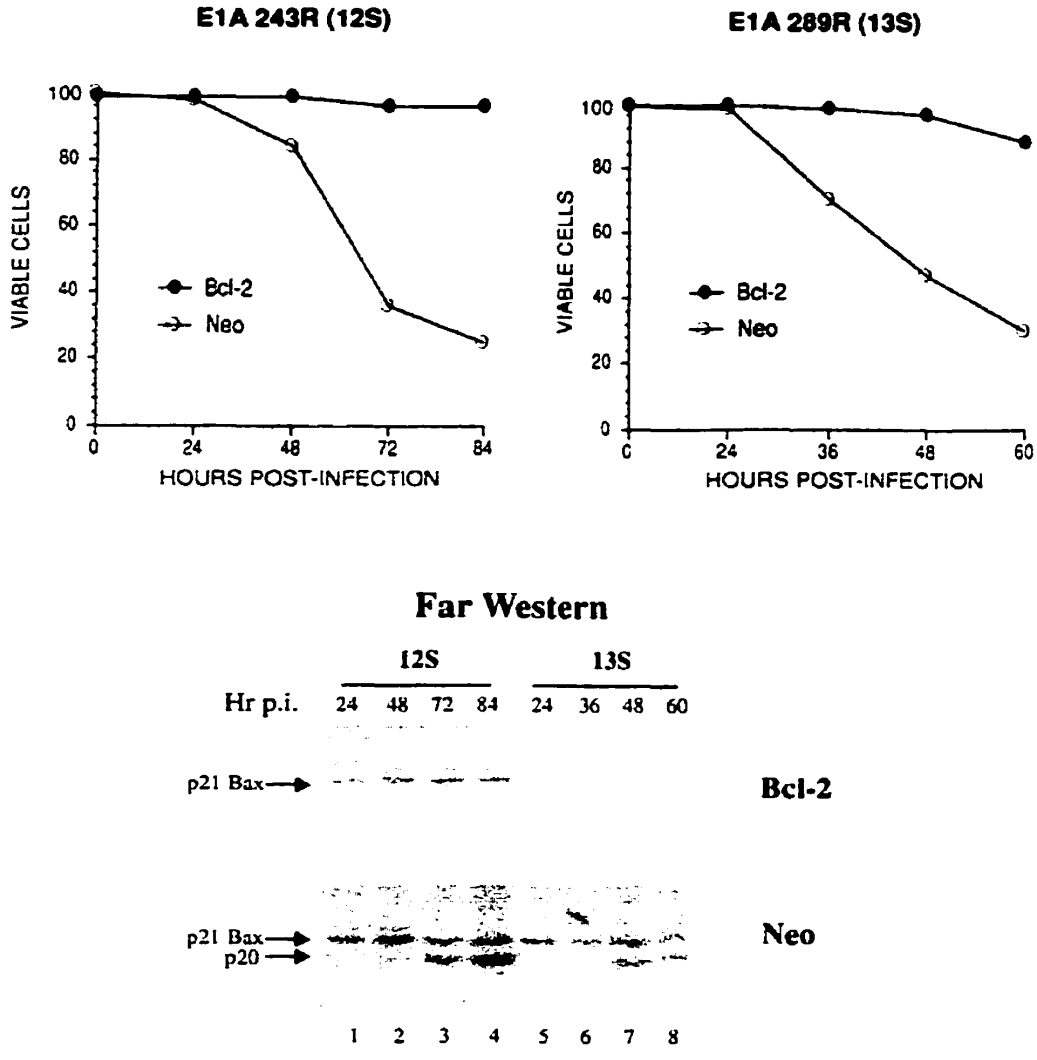

Figure 1. Appearance of a Bcl-2–interacting polypeptide during E1A-induced apoptosis. KB cells expressing neomycin resistance, either alone (*Neo*) or together with Bcl-2, were infected with either adenovirus *dl*520E1B$^-$ (expressing 12S E1A and no E1B products) or *pm*1760/2072 (expressing 12S and 13S E1A but not E1B 19K). At the indicated times after infection, samples of cells were either assessed for viability by exclusion of trypan blue (*graph*) or prepared for ligand blot (*Far Western*) analysis, as described in Materials and Methods, using ^{32}P-Bcl-2Δc21/his6/HMK as a probe (*upper panel*, Bcl-2–expressing cells; *lower panel*, Neo control cells). Ligand blots were visualized by phosphorimaging. The radioactive band associated with a polypeptide of M_r 20 kD is labeled p20, whereas that which comigrates with Bax is designated p21 Bax. The latter was determined using a blot cut along the vertical midline of a protein lane and developing one half by immunoblot analysis with anti–human Bax (Chen et al., 1996) and the other by ligand blotting with ^{32}P-Bcl-2Δc21/his6/HMK (results not shown).

number Z31696), suggesting that p20 derives from the NH_2 terminus of this 27,991-kD (p28) protein. Reverse transcription-PCR analysis of the p28 coding region, using total RNA obtained from KB cells after induction of apoptosis, showed no evidence that p20 arose by differential splicing of p28 mRNA (data not shown). As demonstrated below, Bcl-2 also associates with full length p28 in vitro and in vivo; failure to observe this interaction in the original ligand blot analyses (Fig. 1) was likely the result of relatively inefficient transfer of p28 to nitrocellulose blots.

p28 is identical to a protein previously described as Bap31 and CDM, which is ubiquitously expressed (Adachi et al., 1996). CDM was discovered because of its proximity to the adrenoleukodystrophy locus (Mosser et al., 1994), and Bap31 because it was one of several polypeptides that were found in immunoprecipitates of the B cell receptor complex obtained from detergent-solubilized cells (Kim et al., 1994; Adachi et al., 1996). Another protein present in these precipitates, Bap32, is a homologue of the rat protein prohibitin (McClung et al., 1989). It is noteworthy, however, that prohibitin has recently been localized to the mitochondrial periphery in transfected BHK cells (Ikonen et al., 1995) and that Bap31 (p28) is largely restricted to the ER in rat liver hepatocytes (see below), whereas the B cell receptor is located in the plasma membrane.

p28 Bap31

Fig. 2 *C* highlights several predicted motifs in the human p28 sequence. There are three potential transmembrane (TM) segments (Kyte and Doolittle, 1982) located in the NH_2-terminal half of the molecule. TM1 and TM3 each contain charged residues. Additionally, two potential caspase cleavage sites comprised of identical P1–P4 tetrapeptide recognition sequences (Ala-Ala-Val-Asp) plus a preferred small amino acid (Gly) in the P1′ position are located at positions 164 and 238 in the polypeptide, on either side of a predicted leucine zipper domain (Fig. 2 *C*) and overlapping weak homology to death effector domains found in such proteins as procaspase-8/10 and FADD (Fig. 2 *D*). Cleavage at the proximal caspase recognition site would generate a product (calculated M_r of 18.8 kD) similar in size to p20. Interestingly, the distal caspase recognition site is lacking in the mouse sequence (Fig. 2 *D*). Finally,

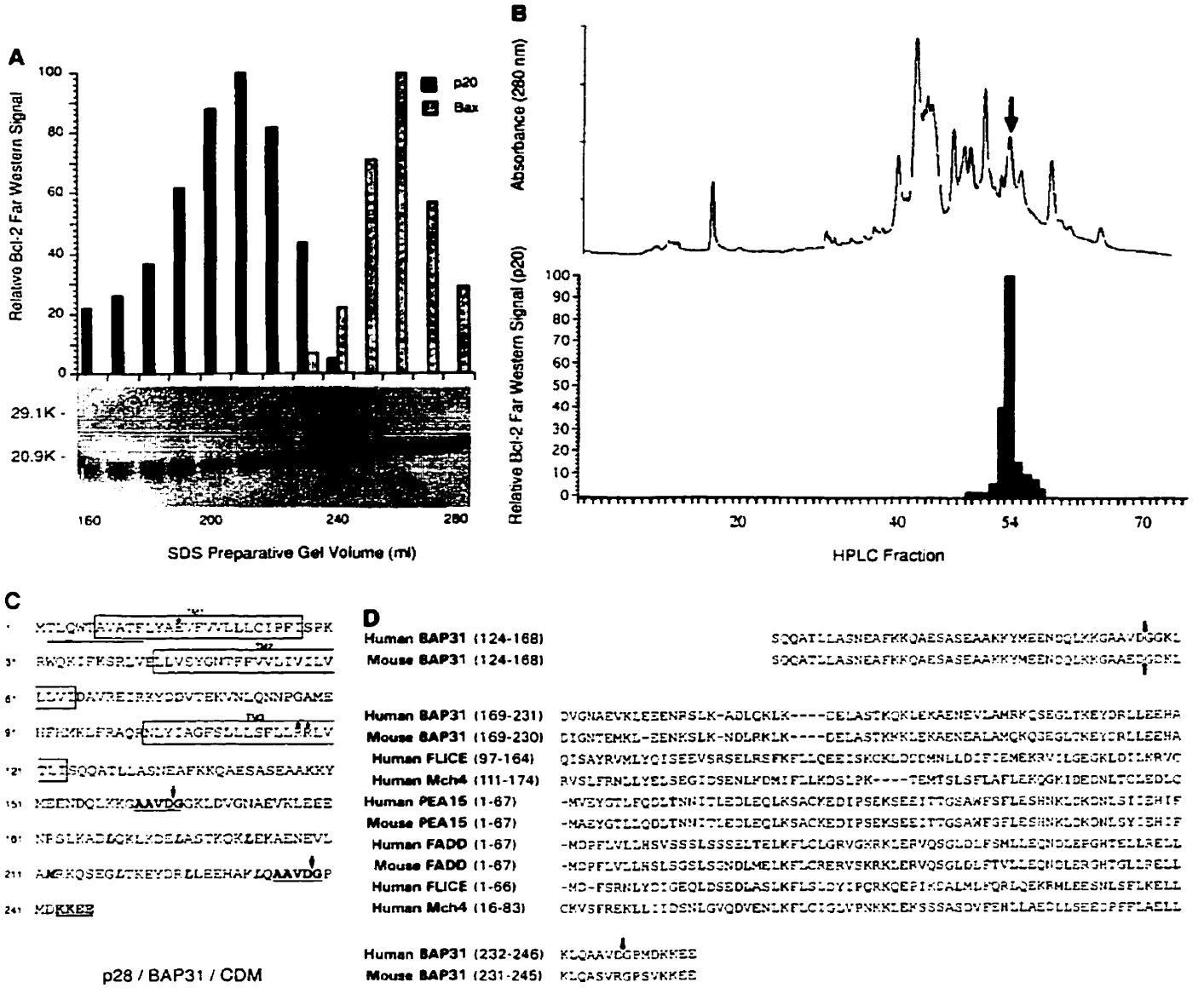

Figure 2. Identification of p20. (*A*) Preparative SDS-PAGE of differentially solubilized protein from KB cells 60 h after infection with adenovirus *pm*1760/2072. Aliquots of fractions eluted from the gel were assayed by ^{32}P-Bcl-2Δc21/his6/HMK Far Western, and the radioactive bands corresponding to p20 and p21 Bax were detected and quantified by phosphorimaging. The levels relative to the maximal signal detected (set at 100) were plotted as a bar graph (*upper panel*). Equal aliquots from the same fractions were also subjected to analytical 12% SDS-PAGE, and the gels were stained with Coomassie brilliant blue (*lower panel*). The positions of molecular mass marker proteins are indicated. *black*, p20; *gray*, Bax. (*B*) Proteins eluting from preparative SDS-PAGE between 190 and 220 ml were concentrated and resolved by reverse-phase HPLC. The upper panel shows the A_{280} profile. Equal aliquots from all fractions were assayed for ^{32}P-Bcl-2Δc21/his6/HMK–interacting protein by Far Western, for which only p20 was detected. Amounts relative to the maximal signal detected (set at 100) were plotted as a bar graph (*lower panel*). Fractions 52, 53, 54 (peak activity), and 55 were individually subjected to NH_2-terminal peptide sequence analysis. (*C*) Polypeptide sequence of p28 Bap31/CDM (*single-letter code*). Peptide sequencing of p20 revealed a perfect match with amino acids 2–11 of human Bap31(*underlined*; these sequence data available from GenBank/EMBL/DDBJ under accession number X81817). This was the only detectable sequence in fraction 54, was detectable together with other sequences in fraction 53, and was not detected in fractions 52 and 55. Predicted TM segments are boxed and contain charged amino acids in TM1 and TM3 (*asterisks*). The predicted caspase recognition sites, AAVD·G, are highlighted, and cleavage is denoted by arrows following Asp at positions 164 and 238. A potential leucine zipper located between the caspase recognition sites is denoted by bold letters, as is the KKXX ER retention signal at the COOH terminus. (*D*) Comparison of putative death effector domain sequences for the indicated proteins. The sequences, given in the single-letter amino acid code, were obtained from GenBank/EMBL/DDBJ, and their relative positions in the molecule are shown in parentheses. Sequences were aligned using the PILEUP program of the GCG software package and were optimized by spacing (shown as dashes). Identical residues and conserved substitutions that were recorded for at least half of the sequences analyzed are shaded in gray.

the molecule terminates in Lys-Lys-Glu-Glu, which conforms to a canonical KKXX COOH-terminal signal that retains integral ER proteins containing COOH termini exposed to the cytosol within this organelle, preventing their exit into the distal secretory pathway (Jackson et al., 1993).

As shown in Fig. 3, p28 was efficiently inserted cotranslationally into dog pancreas microsomes. In contrast to β-lactamase, which was translocated across the ER membrane and deposited in the lumen as a soluble protein, p28 was recovered as an integral protein after release from the ribosome. Whereas the processed form of β-lactamase was protected from external protease (Fig. 3, lane *4*) and liberated from microsomes by alkaline extraction (Fig. 3, lane *3*), p28 was resistant to alkaline extraction (Fig. 3, lane *7*), and exhibited sensitivity to external protease (Fig. 3, lane *8*), resulting in the generation of proteolytic fragments that would be expected for a multispanning integral protein with an exposed cytosolic domain. Unlike β-lactamase, whose NH_2-terminal signal sequence was removed during translocation (Fig. 3, compare lanes *1* and *4*), processing of p28 was not observed (Fig. 3, compare lanes *5* and *6*), suggesting that insertion into the microsomal membrane is initiated by an uncleaved signal anchor. Though not studied in detail, the observed properties of p28 (Fig. 3), together with predictions for the orientation of transmembrane segments in the ER based on charge difference rules (von Heijne, 1986; Hartmann et al., 1989), suggest a topology for p28 in the ER membrane in which the NH_2 terminus of this triple-spanning polypeptide faces the lumen, leaving an ~13-kD COOH-terminal fragment containing predicted caspase cleavage sites, leucine zipper/death effector-like domain, and ER retention motif exposed to the cytosol (see Fig. 3). Biochemical fractionation and cryoimmunocytochemical electron microscopy confirmed that p28 is predominantly located in the ER in rat hepatocytes (not shown).

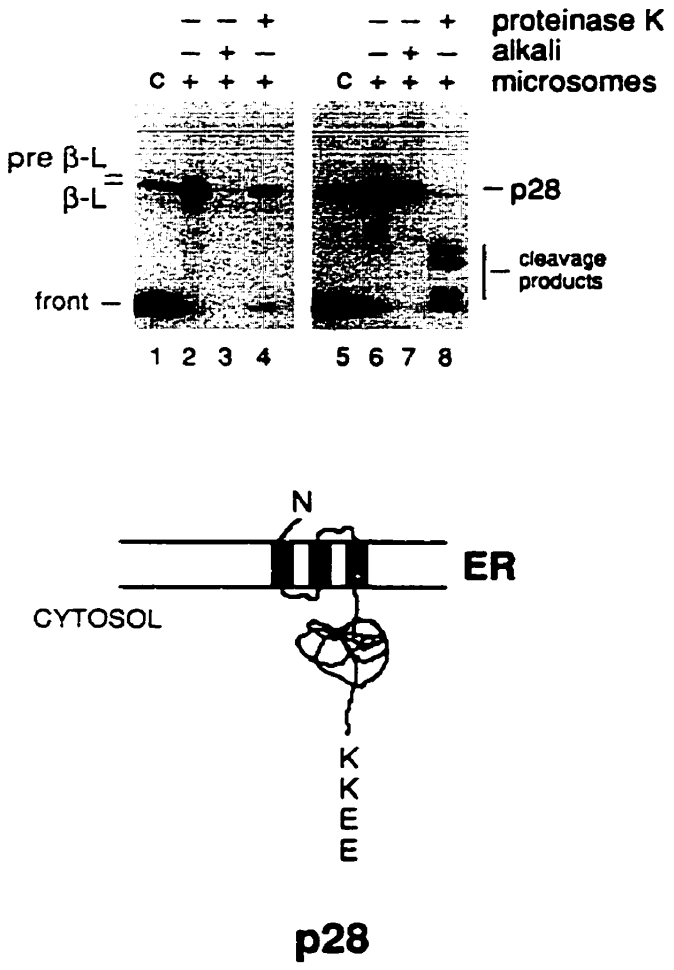

Figure 3. Insertion of p28 into ER microsomes. Pre-β-lactamase (lanes *1–4*) and p28 (lanes *5–8*) mRNA was translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine, in the presence (lanes *2–4* and *6–8*) or absence (lanes *1* and *5*) of ribosome-stripped canine pancreas microsomes (Walter and Blobel, 1983). At the end of the reaction, microsomes were recovered and analyzed by SDS-PAGE and fluorography either directly (lanes *2* and *6*) or after isolation of alkali-insoluble ($NaCO_3$, pH 11.5) product (lanes *3* and *7*; Nguyen et al., 1993), or after treatment with proteinase K (lanes *4* and *8*; McBride et al., 1992). The positions of p28, pre-β-lactamase (*pre-β-L*), and processed β-lactamase (*β-L*) are indicated, as is the gel front. *c*, marker translation product. The schematic shown below the fluorogram depicts the deduced topology of p28 in the ER membrane (see text).

Recombinant p28 and p20 Interact with Bcl-2

Various p28 fusion proteins were constructed in which GST was linked to p28 amino acids 1–246 (full length p28), 1–164 (p20), 122–164, and 165–246. These constructs, together with GST itself, were purified, and equal amounts were examined for their ability to bind to the cytosolic domain of Bcl-2 in a ligand blot assay. As shown in Fig. 4 *A*, reactivity was observed for both GST-p28 (lane *5*) and GST-p20 (lane *4*), with weak activity possibly registering with the COOH-terminal 165–246 amino acid domain (lane *2*), and none detected for the middle 122–164 amino acid domain (lane *3*) or for GST alone (lane *1*).

Bcl-2 Proteins and Procaspase-8 (pro-FLICE) Associate with p28 In Vivo

Epitope-tagged versions of Bcl-X_L, Bcl-2, and wild-type procaspase-8 were expressed separately or in combination in 293T cells, together with either p28-Flag or control Flag. The presence of p28-associated proteins present in immunoprecipitates recovered with anti-Flag was then assessed by immunoblot (Western) analysis (Fig. 4 *B*). To avoid interference with the function of the ER retention signal in p28, the Flag epitope was inserted just upstream of the KKEE motif at the COOH terminus of the protein. As judged by coimmunoprecipitation, Bcl-X_L, procaspase-8, and Bcl-2 each demonstrated a specific association with p28 (Fig. 4 *B*, lanes *3*, *8*, and *14*, respectively). Procaspase-8 was observed as a doublet band that migrated immediately below the Ig heavy chain; transcription-translation of the cDNA in vitro likewise generated a doublet of similar size (not shown). Interestingly, Bcl-X_L and procaspase-8, when expressed in combination, did not mutually antagonize each other's ability to associate with p28 (Fig. 4 *B*, lanes *5* and *10*; note the lower input levels of Bcl-X_L in the cell lysate in lane *5*). Although some activation of wild-type procaspase-8 might be expected, the full length proenzyme

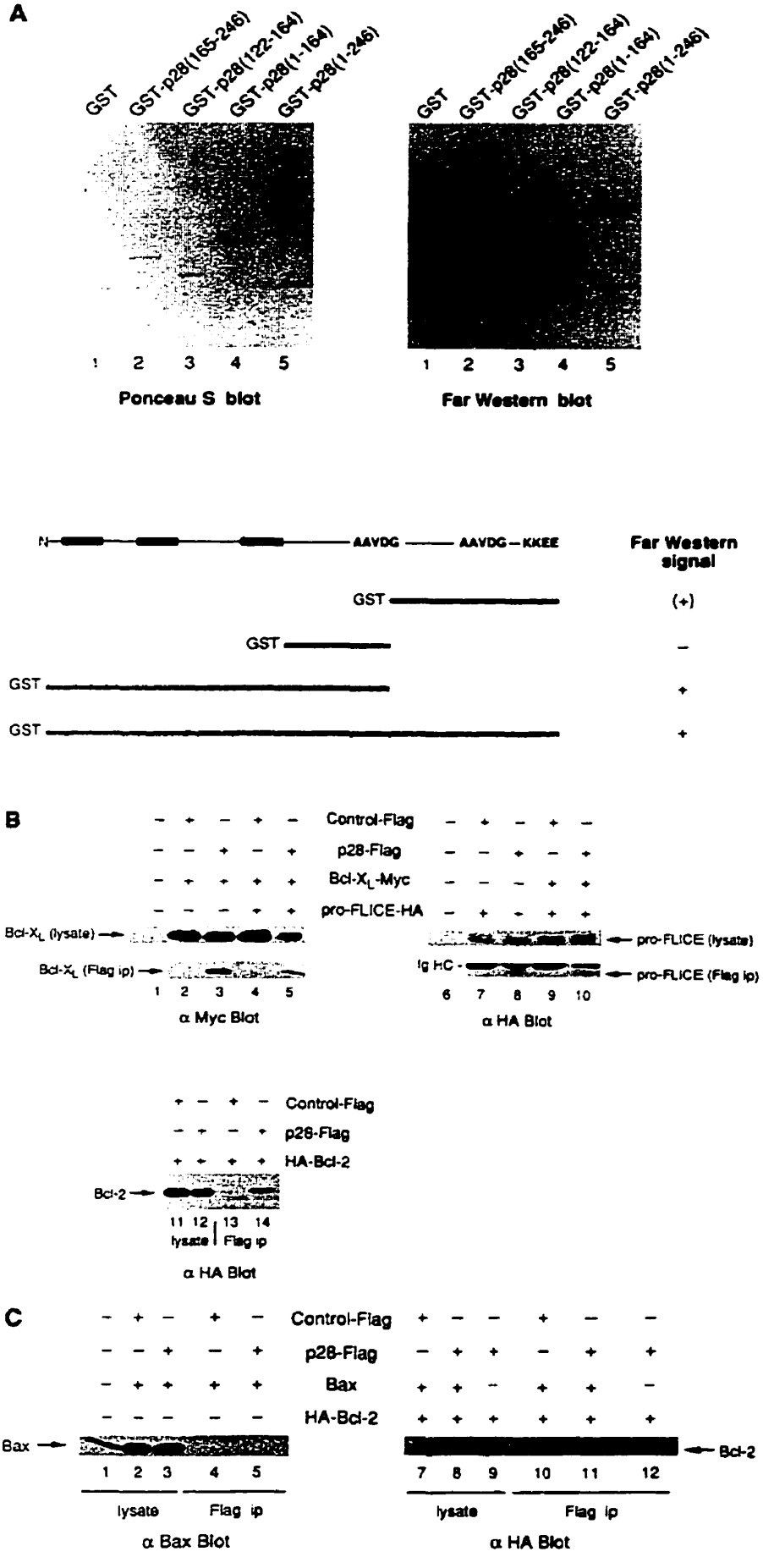

Figure 4. Associations of p28 with Bcl-2, Bcl-X_L, and procaspase-8 (pro-FLICE). (*A*) GST (lane *1*) or GST fused to p28 amino acids 165–246 (lane *2*), 122–164 (lane *3*), 1–164 (lane *4*), and 1–246 (lane *5*) were expressed in bacteria, purified, and transferred to nitrocellulose in duplicate after SDS-PAGE. One blot was stained with Ponceau S and the other probed by ligand blotting (Far Western) with ^{32}P-Bcl-2Δc22/his6/HMK, as indicated. Constructs and results are summarized below the blots. (*B*) Standard recombinant DNA manipulations were used to create cDNAs encoding Bcl-X_L tagged at the COOH terminus with the Myc epitope EQKLISEEDL (Chinnayan et al., 1997); pro-FLICE tagged at the COOH terminus with the hemagglutinin (*HA*) epitope, YPYDVPDYA (Chinnayan et al., 1997); Bcl-2 tagged at the NH_2 terminus with the HA epitope (Nguyen et al., 1994); and p28 tagged with the Flag epitope, in which the Flag sequence MDYKDDDDKA was inserted between Pro240 and Met241 of p28. The recombinant cDNAs, or Flag DNA alone (*Control-Flag*), were inserted into pcDNA 3 (Invitrogen) or RcRSV (Pharmacia Fine Chemicals) (HA-Bcl-2) and transfected into 293T cells, as indicated (*pluses* and *minuses*). After incubation of cell lysates with anti-Flag antibody, immunoprecipitates (*ip*) and lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and the blot developed with the indicated antibody and visualized by enhanced chemiluminescence. *Ig HC*, immunoglobulin heavy chain. (*C*) Same as in *B*, except that Bax was included in cotransfections, as indicated.

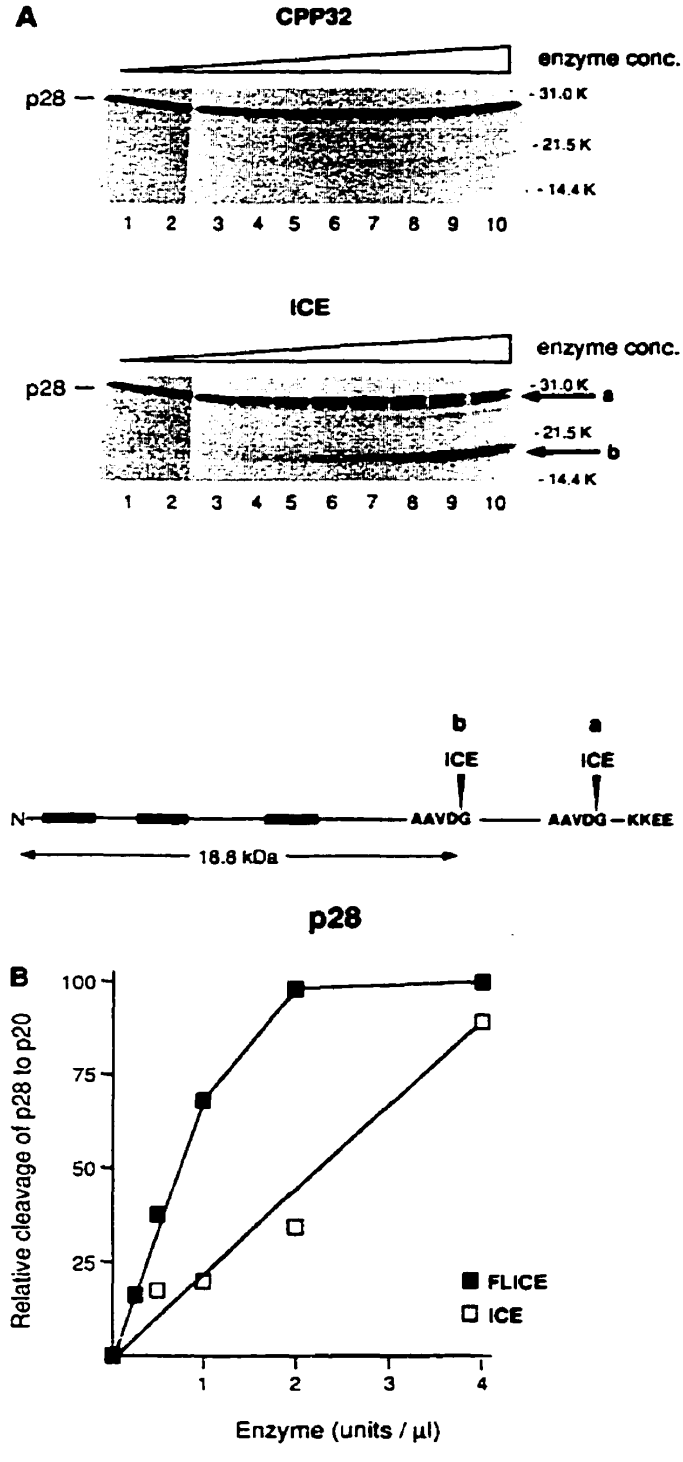

Figure 5. In vitro cleavage of p28 by caspase-1 (*ICE*) and caspase-8 (*FLICE*) but not by caspase-3 (*CPP23*). (*A*) ^{35}S-labeled transcription–translation product of p28 cDNA was incubated with increasing concentrations of CPP32 or ICE, and the products were resolved by SDS-PAGE (Nicholson et al., 1995). Units of enzyme added per 25 µl reaction mixture were: none (lane *1*), 0.0056 (lane *2*), 0.98 (lane *3*), 1.95 (lane *4*), 3.9 (lane *5*), 7.8 (lane *6*), 15.6 (lane *7*), 31.2 (lane *8*), 62.5 (lane *9*), and 125 (lane *10*). The positions of polypeptide molecular mass markers are shown. The arrows designated *a* and *b* denote cleavage products whose sizes are consistent with cleavage of p28 at the sites indicated by *a* and *b* in the schematic at the bottom of the figure. (*B*) Same analysis as in *A*, except that p28 was incubated with purified ICE or FLICE, and the resulting p20 cleavage product was quantified using a Phosphorimager. 1 U of caspase enzyme activity is equivalent to 1 pmol aminomethylcoumarin liberated from fluorogenic tetrapeptide-AMC per min at 25°C at saturating substrate concentrations (Nicholson et al., 1995).

was readily detectable at similar levels in both the presence and absence of Bcl-X_L (Fig. 4 *B*, lanes *8* and *10*).

Finally, the pro-apoptotic member of the Bcl-2 family, Bax (Oltvai et al., 1993), did not coimmunoprecipitate with p28 after their coexpression in 293T cells (Fig. 4 *C*, lane *5*), despite the fact that significant expression levels of transfected Bax were recorded (lanes *2* and *3*). However, Bax prevented Bcl-2 from associating with p28 (Fig. 4 *B*, compare lanes *11* and *12*). Although the level of Bcl-2 in cell lysates was somewhat lower in transfectants containing Bax (Fig. 4 *B*, compare lanes *8* and *9*), such a level of Bcl-2 would otherwise have been sufficient to readily detect coimmunoprecipitation of Bcl-2 and p28.

p28 is Cleaved to p20 by FLICE-related Caspase

To test the possibility that the predicted caspase recognition sequences in p28, AAVD·G, can in fact be recognized by one or more of these enzymes, ^{35}S-labeled p28 transcription–translation product was incubated with increasing concentrations of either caspase-1 (ICE) or caspase-3 (CPP32) in vitro (Nicholson et al., 1995), and the products were examined after SDS-PAGE (Fig. 5 *A*). Little reactivity was observed for caspase-3 over a wide range of enzyme concentration. caspase-1, on the other hand, generated two products (denoted *a* and *b* in Fig. 5 *A*), whose apparent sizes in SDS gels (~27 and ~20 kD, respectively) are consistent with cleavage occurring at both AAVD/G sites. Of note, p28 was more sensitive to cleavage by caspase-8 (FLICE) than by caspase-1 (ICE; Fig. 5 *B*).

Two cleavage products of p28, similar in size to those seen in vitro, were also observed in cells that had been induced to undergo apoptotic cell death in response to infection by 19K-defective adenovirus (Fig. 6). In Fig. 6 *A*, p28 cleavage during apoptosis in vivo was analyzed using antibodies raised in chicken to either of two regions of the protein: p28 amino acids 122–164 (α p28-M) and 165–246 (α p28-C). Cleavage products were detected with α p28-M but not with α p28-C, a finding consistent with the suggestion from peptide sequence analysis that the p20 cleavage product derives from the NH_2 terminus of p28 (Fig. 2). α p28-C also failed to detect the larger of the two cleavage products (designated *a* in Fig. 6 *A*) despite the predicted overlap of this product with the sequence injected into chickens. Presumably, this means that the extreme eight amino acids of p28 are critically important for epitope recognition by this antibody. Finally, protein electrophoretic blots were developed from apoptotic cell extracts, cut in half along the vertical midline of a protein lane, and one half probed with α p28-M and the other with ^{32}P-Bcl-2Δc21/his6/HMK. p20 detected by the Bcl-2 probe migrated exactly with p20 detected by α p28-M immunoblotting (not shown).

In Fig. 6 *B*, the effect of Bcl-2 on the appearance of p28 cleavage products after cell infection with 19K-deficient

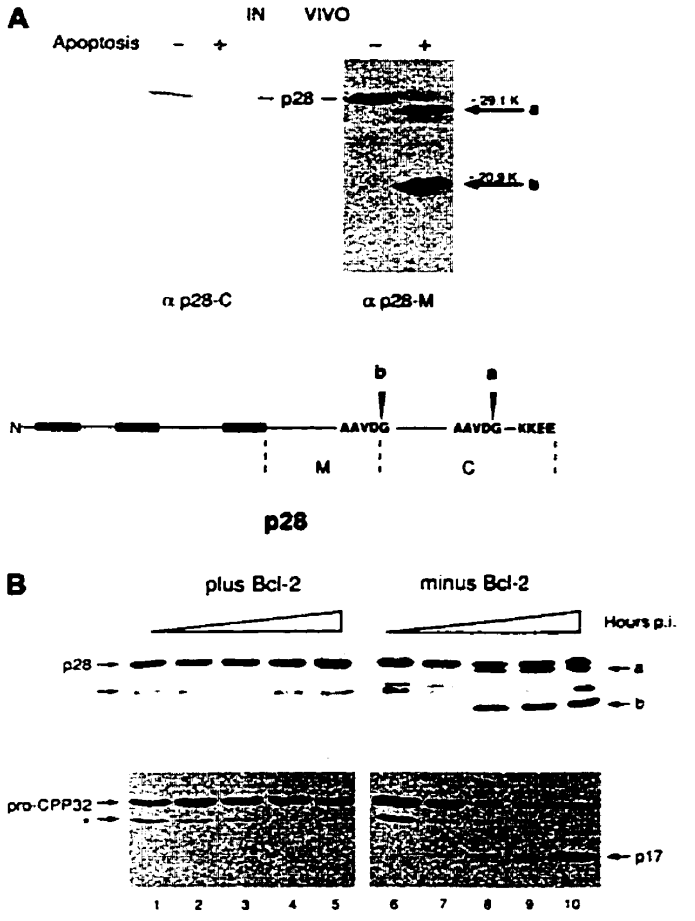

Figure 6. Induction of p28 cleavage and procaspase-3 (pro-CPP32) processing during apoptosis in vivo. (*A*) Cell extracts were obtained from KB cells that had either been infected for 60 h with adenovirus *pm*1716/2072 lacking expression of E1B 19K or had been mock infected (+ *or* − *Apoptosis*, respectively). After 12% SDS-PAGE and transfer to nitrocellulose, blots were incubated with affinity-purified chicken antibody against p28 amino acids 165–246 (*α p28-C*) or p28 amino acids 122–164 (*α p28-M*) and were developed with secondary antibody conjugated either to HRP and visualized by electrochemiluminescence (Amersham Intl., Arlington Heights, IL) (*α p28-M*) or to alkaline phosphatase and visualized with NBT/BCIP (Boehringer Mannheim Biochemicals, Indianapolis, IN) (*α p28-C*), according to the manufacturer's instructions. Bands corresponding to p28 are indicated. Arrows labeled *a* and *b* denote products whose sizes are consistent with cleavage of p28 at the sites designated *a* and *b* in the schematic. (*B*) KB cells expressing neomycin resistance either alone (*minus Bcl-2*, lanes *6–10*) or together with Bcl-2 (*plus Bcl-2*, lanes *1–5*) were infected with adenovirus *pm*1716/2072 lacking expression of E1B 19K and cell extracts prepared at 0, 24, 36, 48, and 60 h postinfection (*p.i.*; lanes *1* and *6*, *2* and *7*, *3* and *8*, *4* and *9*, and *5* and *10*, respectively). Aliquots (15 μg protein) were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and blots were probed with antibody against p28-M or against the 17-kD subunit of CPP32 (Boulakia et al., 1996), and the products were developed as described in *A*. The positions of p28 and the cleavage products *a* and *b* are indicated in the upper panels. The arrow denotes a cross-reacting product whose appearance is variable (e.g., it did not appear in *A*). The positions of full length pro-CPP32 and the processed 17-kD subunit (p17) and putative 29-kD processing intermediate (*asterisk*) are indicated in the lower panels.

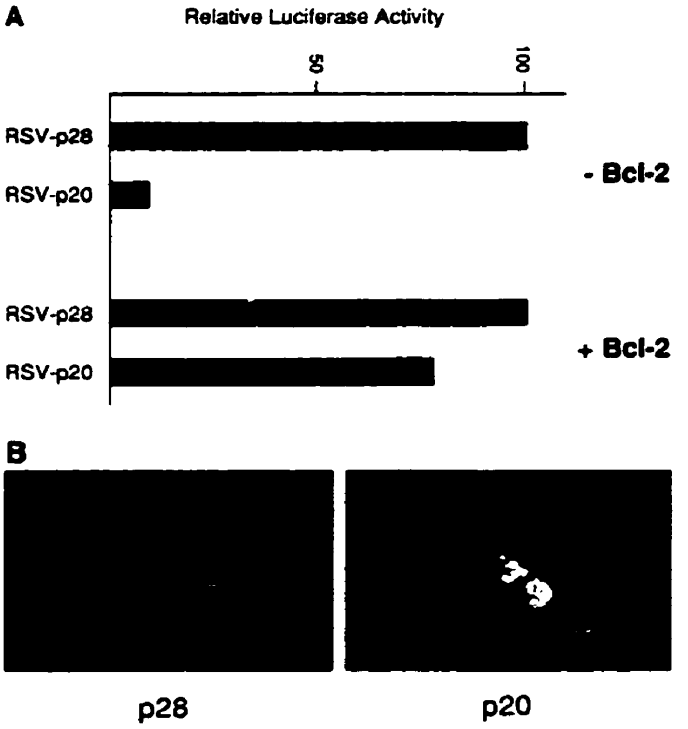

Figure 7. Ectopic expression of p20 induces apoptosis. (*A*) CHO LR73 cells expressing neomycin resistance, either alone (− *Bcl-2*) or together with Bcl-2 (+ *Bcl-2*), were cotransfected with a luciferase reporter plasmid and Rc/RSV expressing either full length p28 or p28 amino acids 1–164 (i.e., p20). After 2 d, cells were recovered, analyzed for luciferase activity, and the enzyme activity expressed relative to the values obtained in the presence of p28 (arbitrarily set at 100). The results shown are the average of two separate experiments. (*B*) CHO cells were transfected with the p28 and p20 expression plasmids together with pHook (Invitrogen). 24 h later, transfected cells were recovered with Capture-Tec beads, cultured on coverslips, stained with 4′,6′-diamidino-2-phenyl indole (DAPI), and visualized under a microscope.

adenovirus was examined using the α p28-M antibody. In the absence of Bcl-2 expression, the time course of appearance of these products closely followed the time course for activation of procaspase-3 (CPP32), as judged by processing of the pro-enzyme to the p17 catalytic subunit (Fig. 6 *B*, lanes *6–10*). However, both p28 cleavage and procaspase-3 processing were blocked in virus-infected cells that express Bcl-2 (Fig. 6 *B*, lanes *1–5*).

Ectopic Expression of p20 Induces Apoptosis

CHO (*neo*) cells were transiently cotransfected with a luciferase reporter gene together with RcRSV expressing either p28 or p20. Subsequent measurements revealed that coexpression of p20 with the reporter severely depressed the amount of luciferase activity obtained relative to coexpression with p28 (Fig. 7 *A*). p28, on the other hand, had no deleterious effect on the recovery of luciferase activity compared to a control RcRSV plasmid that did not encode protein (not shown). If these same transfections were conducted in cells stably expressing Bcl-2, however, Bcl-2 largely

overcame the dominant negative influence of p20 on luciferase activity (Fig. 7 *A*), presumably because p20 could no longer interfere with normal p28 function. Because of this protective effect by Bcl-2, we conclude that the negative influence of p20 on luciferase activity was the result of induction of apoptosis. This was confirmed by microscopic analysis, which revealed apoptotic nuclei in p20- but not p28-transfected cells (Fig. 7 *B*). The findings described in Fig. 7 have been consistently observed many times and in different cell types.

Discussion

We have identified p28 Bap31 as a component of a putative apoptotic signaling complex in the ER that also includes Bcl-2/Bcl-X_L and procaspase-8 (pro-FLICE). At least with respect to recruitment of procaspase-8, the function of the p28 complex may be analogous to Fas and TNFR-1, apoptotic receptors in the plasma membrane that respond to FasL and TNF, respectively, by activating receptor-associated procaspase-8 and initiating the caspase cascade (for review see Nagata, 1997). Association of procaspase-8 with these receptor complexes is achieved through interactions of its death effector domain with an analogous domain in the adaptor molecule FADD (Boldin et al., 1996; Muzio et al., 1996). Despite the fact that p28 contains a weak death effector homologous domain within its cytosolic region, however, we find no evidence that p28 and procaspase-8 interact directly, at least based on binding assays in vitro (not shown). Thus, evidence for an autonomously functional death effector domain in p28 is lacking. The observed association of procaspase-8 with p28 in vivo, therefore, likely depends on an as yet unidentified adaptor component within the p28 complex. Expression of the adenovirus E1A oncoprotein presumably results in activation of procaspase-8, as judged by the ensuing rapid and specific cleavage of p28 in vivo at sites that exhibit a marked substrate preference by purified caspase-8 in vitro. Such a claim is consistent with the finding that CrmA, an inhibitor of FLICE-related caspases but not of caspase-3 (Srinivasula et al., 1996; Zhou et al., 1997), significantly retards the onset of E1A-induced apoptosis in viral infected cells (our unpublished data). It remains to be determined, however, if E1A results in activation of the procaspase-8 (or related caspase) members that are directly associated with the p28 complex. Whichever the case, p28 is cleaved to p20 after E1A expression, and the p20 molecule itself can induce cellular apoptotis. p20 may act either to ampilify a caspase-8–initiated protease cascade (Srinivasula et al., 1996; Muzio et al., 1997) or to contribute to a parallel pathway. Significantly, Bcl-2 blocks the appearance of p20 after E1A expression. It remains to be determined whether this is because Bcl-2 subverts the E1A signal that results in activation of procaspase-8 or because it protects p28 against activated caspase-8. Bax, a pro-apoptotic member of the Bcl-2 family, does not appear to associate with the p28 complex. However, it blocks Bcl-2 from doing so, presumably because competing interactions between Bcl-2 and Bax (Oltvai et al., 1993) prevent interactions between Bcl-2 and p28.

There is a growing body of evidence that mitochondria may play an important role in apoptotic signaling leading to downstream activation of caspase-3 and other effector proteases (Kroemer et al., 1997; Kluck et al., 1997; Yang et al., 1997). Moreover, these or other mitochondrial events may be controlled by phosphorylation (Wang et al., 1996*a*; Zha et al., 1996) which in turn may be influenced by Bcl-2 (Wang et al., 1996*a*). Under normal conditions, Bcl-2 and Bcl-X_L are localized to both the mitochondrial outer membrane and ER, suggesting that events influencing these proteins may apply to both membrane sites. Nevertheless, replacing the COOH-terminal Bcl-2 signal anchor with either a heterologous mitochondrial outer membrane signal anchor (Mas70p; McBride et al., 1992) or a posttranslational ER-specific insertion sequence (aldehyde dehydrogenase; Masaki et al., 1994) can restore anti-apoptotic activity to the protein (Nguyen et al., 1994; our unpublished data). Similar results have been obtained with other mitochondrial- and ER-specific targeting sequences (Zhu et al., 1996). These findings are consistent with the idea that ER and mitochondria may cooperate to elicit apoptotic signals, and that regulation of these signals by Bcl-2 at either compartment may be effective under certain conditions. Alternatively, apoptotic regulatory systems at the two membrane sites may respond differentially to individual inducers. Although the potential role for Ca^{2+} in apoptosis remains unresolved, the ability of mitochondria to decode ER-transmitted oscillating Ca^{2+} signals (Hojnóczky et al., 1995; Camacho and Lechleiter, 1995; Jouaville et al., 1995) is one example in which the two organelles clearly cooperate to regulate metabolic events. Consistent with a role for calcium signaling in apoptosis is the recent finding that inhibition of the type I inositol 1,4,5-triphosphate receptor calcium release channel in the ER confers resistance to diverse apoptotic stimuli. It remains to be determined whether or not p28 is involved in either this or another ER–mitochondrial program. If so, however, the fact that predicted transmembrane segments 1 and 3 contain positively charged residues implies that p28 may be part of a larger complex that thermodynamically stabilizes these residues within the membrane lipid bilayer. Such a structure might also be compatible with the predicted pore-forming properties of the Bcl-X_L (Minn et al., 1997) and Bcl-2 (Schendel et al., 1997) cytosolic domains and contribute to p28–Bcl-2 interactions.

Finally, genetic studies in *Caenorhabditis elegans* have identified and ordered a core machinery for regulation of cell death in which the Bcl-2 homologue, Ced-9, prevents Ced-4 from activating the ICE-like caspase Ced-3 (Hengartner et al., 1996; Shaham and Horvitz, 1996). Reconstitution of these events in mammalian cells has recently been achieved (Chinnaiyan et al., 1997; Wu et al., 1997) and has revealed that Ced-4 can mechanically link the initiator procaspase-8 and -1 pro-enzymes with Bcl-X_L (Chinnaiyan et al., 1997). Moreover, Ced-4 can compete for interaction of procaspase-8 with the predicted endogenous molecule(s) in mammalian cells that bridges this Bcl-2 protein with procaspase-8. Ced-4, like the cytosolic tail of p28, may contain a weak death effector homologous domain (Bauer et al., 1997). These findings all conform with the predicted function of the putative p28 apoptotic signaling complex described here. Moreover, they raise the possibility that the mammalian homologue of Ced-4, if one exists, may be a constituent of this complex.

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Connecting Text

In the proceeding chapter, a Ced-4-like adaptor molecule was determined to be a constituent of the p28Bap31 complex. P28Bap31 can associate simultaneously with both Bcl-2/Bcl-X_L and procaspase-8 *in vivo*. However, the result obtained from *in vitro* binding studies indicated that, unlike Bcl-2, procaspase-8 did not interact with p28bap31 directly. Therefore, it is likely that an adaptor molecule may mediate the interactions between these proteins *in vivo*. In co-transfected cells, Ced-4 mechanically links Bcl-X_L to procaspase-8 suggesting the existence of mammalian Ced-4-like molecule(s). It also raises the possibility that the adaptor molecule in the p28Bap31 complex is related to the mammalian Ced-4-like proteins. The following chapter describes our efforts to show that Ced-4 interacts with p28Bap31 and that a Ced-4-like adaptor is a part of p28Bap31 complex.

Chapter III

Bcl-X_L Cooperatively Associates with the Bap31 Complex in the Endoplasmic Reticulum, Dependent on Procaspase-8 and Ced-4 Adaptor

Summary

Bap31 is a polytopic integral membrane protein of the endoplasmic reticulum and forms a complex with Bcl-2/Bcl-X_L and procaspase-8 (Ng et al. (1997) *J Cell Biol.* 139, 327-338). In co-transfected human cells, procaspase-8 is capable of interacting with Ced-4, an important adaptor molecule in *C. elegans* that binds to and activates the *C. elegans* procaspase, proCed-3. Here, we show that the predicted death effector homology domain within the cytosolic region of Bap31 interacts with Ced-4 and contributes to recruitment of procaspase-8. Bcl-X_L, which binds directly but weakly to the polytopic transmembrane region of Bap31, indirectly and cooperatively associates with the Bap31 cytosolic domain, dependent on the presence of procaspase-8 and Ced-4. Ced-4Δc does not interact with Bcl-X_L but rather displaces it from Bap31, suggesting that an endogenous Ced-4-like adaptor is a normal constituent of the Bap31 complex and is required for stable association of Bcl-X_L with Bap31 in vivo. These findings indicate that Bap31 is capable of recruiting essential components of a core death-regulatory machinery.

INTRODUCTION

Genetic studies in the nematode *Caenorhabditis elegans* have identified and ordered a core machinery for regulation of apoptotic programmed cell death in which the Bcl-2 homolog, Ced-9, prevents Ced-4 from activating the ICE-like caspase, Ced-3, and thus blocks ensuing cell death (1-4). Recent reconstitution of these events both in vitro and in heterologous yeast and human cells has revealed that Ced-9 directly binds and sequesters Ced-4 (5-9), which in turn remains associated with Ced-3 (6,9). This presumably prevents the Ced-4 adaptor from triggering autocatalytic processing and activation of Ced-3 (10,11). Of note, when expressed in human cells, Ced-4 is also capable of mechanically linking the Bcl-2 family protein, Bcl-X_L, to procaspase-8 (6). Ced-4 likely achieves this by substituting for an endogenous Ced-4-like adaptor molecule that otherwise would connect Bcl-X_L and procaspase-8 (6). Caspase-8 (12-14) belongs to the initiator class of caspases (14-16) whose members appear to function upstream of mitochondria (17) to activate the death pathway. Significantly, however, Ced-4 itself does not associate with procaspase-3 (6), a downstream effector caspase whose activation may depend on a combination of mitochondrial-released factors and the Ced-4-like cytosolic protein, Apaf-1 (18; reviewed in ref. 19). A major question, however, is the mechanism by which these Ced-4 controlled events are linked to the plethora of signals that result in activation of caspases and subsequent cell death.

Recently, we identified a Bcl-2/Bcl-X_L and procaspase-8 associated protein in the endoplasmic reticulum (ER[1]), p28 Bap31 (20). Bcl-2 family proteins are located in the ER / nuclear envelope and mitochondrial outer membrane (21-23) and, in the latter location, appear to prevent activation of

downstream effector caspases such as procaspase-3 in response to diverse death signals (see 19 and 24). Initiator caspases such as procaspase-8, on the other hand, are well characterized constituents of the Fas and TNFR1 apoptosis signaling complexes in the plasma membrane (25-27). These complexes, however, are highly restricted in the death signals to which they respond and are not directly influenced by Bcl-2 family proteins . The ability of Bap31 to associate with both procaspase-8 and Bcl-2/Bcl-X_L, therefore, raises the possibility that the Bap31 complex in the ER might cooperate with events in the mitochondrion to control proximal and distal steps in a Bcl-2 regulated caspase cascade. If so, the ability of Ced-4 to bridge Bcl-X_L and procaspase-8 (6) predicts that a Ced-4-like adaptor molecule may also be a part of the Bap31 complex.

EXPERIMENTAL PROCEDURES

Plasmids - cDNAs encoding proteins tagged with a specific epitopes were constructed in expression vectors. Flag epitope was inserted toward the C-terminus of Bap31, immediately upstream of the KKEE ER retrieval signal; Myc and HA epitopes were placed at the C-termini of Bcl-X_L and proFLICE, respectively. Details are provided in ref. 20. Standard recombinant DNA methodology was employed to create pcCDNA3.1 vectors (Invitrogen) encoding Bap31ΔN119, lacking amino acids 2-119, and Bap31Δ167-240, lacking amino acids 167-240. Authenticity of all constructs was validated by sequence analysis. Similarly, pGEX 2T vectors (Invitrogen) encoding GST and GST-Bap31ΔN119 were employed to express the proteins in bacteria, which were purified as described in refs. 20 and 30. The coding region of ced-4S (28) was obtained by reverse transcription (RT) PCR using total *C. elegans* RNA and 5'-CGAGGTACCATGCTCTGCGAAATCGA-3' as the sense primer and 5'CGAGGTACCTCAAGCGTAATCTGGAACATCGTAT GGGTAACAGCATGCAAAATTTTTG-3' as antisense primer, and the construct inserted into pCDNA3.1 by standard manipulations. RT PCR was employed to delete the region encoding amino acids 303-548, using 5'-CTAGAATACCCATACGATGTTCCAGATTACGCTTAAGGTACCGC-3' as sense primer and 5'GGCCGCGGTACCTTAAGCGTAATCTGGAACA TCGTATGGGTATT-3' as antisense primer.

Transfections and immunoprecipitation - Details concerning transfection of expression vectors into human 293T cells, subsequent preparation of cell lysates, and analysis of products by immunoprecipitation followed by transfer to nitrocellulose for immunoblot analysis are given in ref.20.

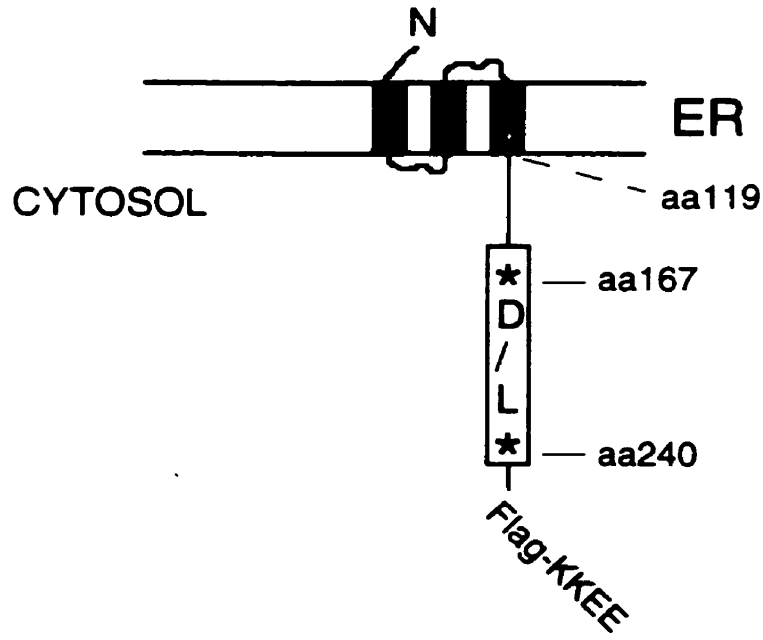
RESULTS AND DISCUSSION

The predicted death effector homology domain in Bap31 (amino acids 265-238) is flanked on either side by sites that are cleaved by caspase-8 or related caspase during adenovirus E1A-induced apoptosis (20) (summarized in Fig. 1A). The resulting p20 Bap31 product is a potent inducer of apoptosis when expressed ectopically in otherwise normal cells, presumably because it has a dominant-negative effect on endogenous Bap31(20). In vitro mapping has revealed that Bcl-2 proteins bind directly to the N-terminal domain of Bap31, which includes the polytopic membrane-associated region, and weakly if at all with the cytosolic domain (20). As expected, therefore, full-length Flag-tagged Bap31, but not the Bap31 cytosolic domain alone (Bap31ΔN119), interacted with Bcl-X_L in co-transfected human 293T cells, as judged by co-immunoprecipitation with anti-Flag antibody (Fig. 1B, compare lanes 3 and 4). However, when Bap31ΔN119 was coexpressed with both Bcl-X_L and procaspase-8 (proFLICE), a strong association between Bcl-X_L and the Bap31 cytosolic domain was recorded (lane 5). Thus, Bcl-X_L may be tethered at the N-terminus of Bap31 and associate indirectly and cooperatively with the Bap31 cytosolic domain, dependent on the presence of procaspase-8. Failure to achieve reconstitution of these interactions *in vitro* (not shown), however, suggested that an adaptor molecule might be required.

As a preliminary step indicating that Ced-4 might associate with Bap31, a transcription-translation product of the proapoptotic short splice-form of Ced-4 (28) was found to interact in vitro with a glutathione S-transferase fusion protein containing the Bap31 cytosolic domain (GST-Bap31ΔN119) (Fig. 2). Furthermore, when Ced-4 was co-expressed with Bap31-Flag in 293T cells and total cell extracts were incubated with anti-

Figure 1. Procaspase-8 (proFLICE) promotes an indirect association of Bcl-X_L with the cytosolic domain of Bap31. *A*, Topology and domain structure of Bap31. The orientation of the three transmembrane segments of Bap31 predicts a N_{lumen}-C_{cyto} topology in the ER membrane (20). The region of the 13 kDa cytosolic domain containing putative death effector homology (D) and leucine zipper (Z) domains, flanked on either side by caspase-8 recognition sites (asterisks), are boxed. Amino acid positions used for generating deletion mutants are indicated. Flag epitope was inserted immediately upstream of the C-terminal KKEE ER retrieval signal (20). *B*, Cooperative association of Bcl-X_L and procaspase-8 (proFLICE) with the Bap31 cytosolic domain. Human embryonic kidney 293T cells were transfected with vectors encoding the indicated epitope-tagged proteins (20), and total cell extracts (lysate) containing similar levels of either Bap31-Flag or Bap31ΔN119-Flag were prepared 30 h later; immunoprecipitations (ip) obtained with mouse M2 anti-Flag antibody (IBI-A Kodak) were resolved by 12% SDS PAGE, blotted onto nitrocellulose, and developed with anti-Myc 9E10 antibody (Babco) to detect Bcl-X_L, which was visualized by enhanced chemoluminescence. Experimental details are provided in ref. 20.

A


N
ER
CYTOSOL
aa119
*
D
/
L
*
aa167
aa240
Flag-KKEE

B

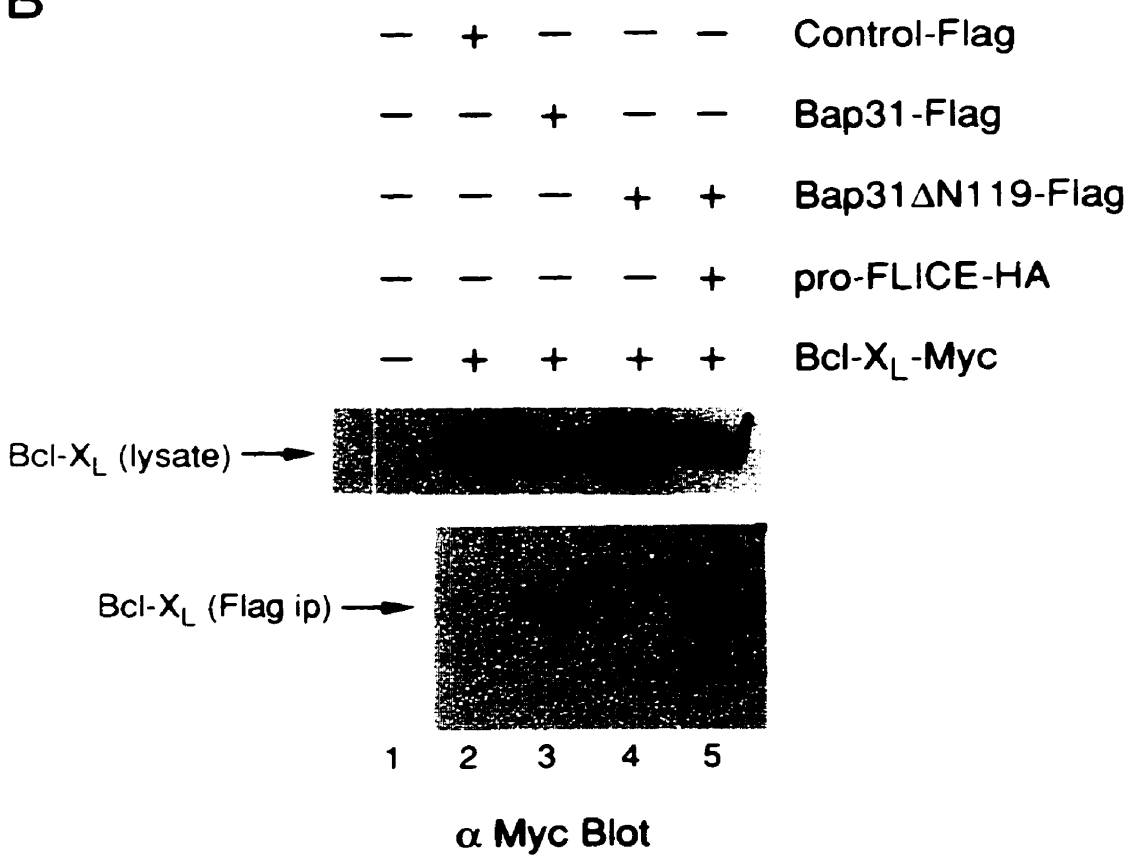
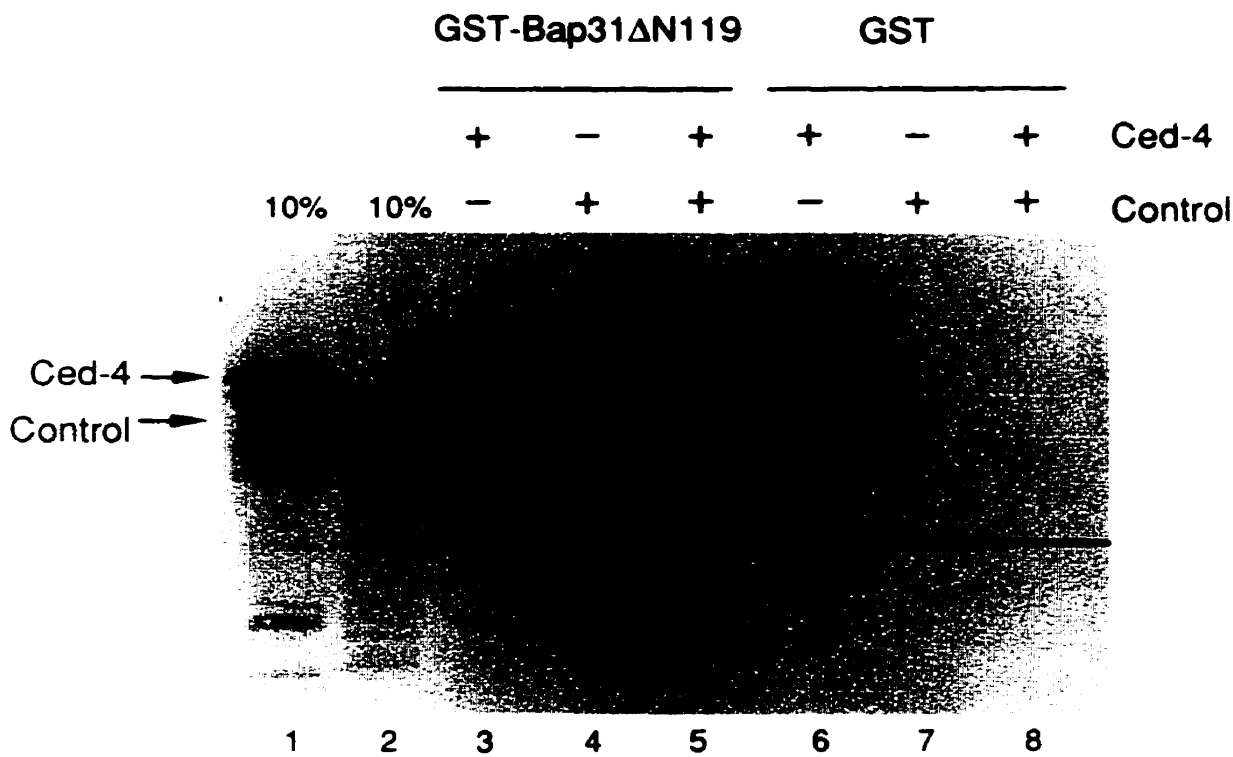

— + — — — Control-Flag
— — + — — Bap31-Flag
— — — + + Bap31ΔN119-Flag
— — — — + pro-FLICE-HA
— + + + + Bcl-XL-Myc
Bcl-XL (lysate)
Bcl-XL (Flag ip)
1 2 3 4 5
α Myc Blot

Figure 2. Binding of Ced-4 to the Bap31 cytosolic domain *in vitro*. cDNAs encoding Ced-4 or Control protein were constructed in Bluescript and the ^{35}S-labeled proteins generated by transcription-translation in rabbit reticulocyte lysate, as described (30). Following incubation with equivalent amounts of GST (lanes 5 and 6) or GST-Bap31ΔN119 (lanes 3 and 4) linked to glutathione Sepharose, the associated translation product was recovered and visualized by SDS PAGE and fluorography, as described (20,30). Lanes 1 and 2, 10% of input Ced-4 and Control protein, respectively.

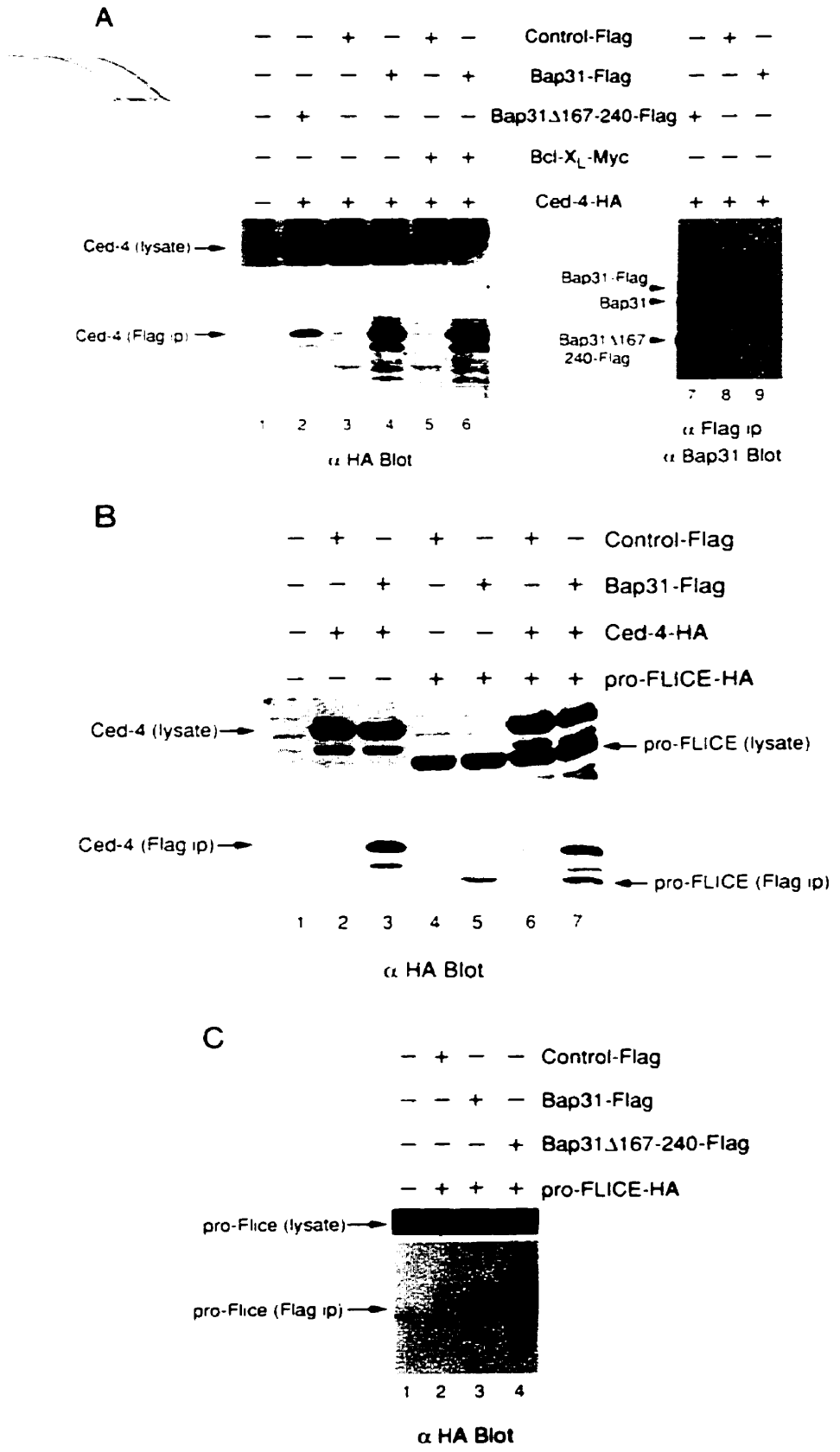

GST-Bap31ΔN119
GST
+ − + + − + Ced-4
10% 10% − + + − + + Control
Ced-4
Control
1 2 3 4 5 6 7 8

Flag antibody, Ced-4 co-immunopreciptated with Bap31-Flag but not with Control-Flag (Fig. 3A, lanes 3 and 4). This association was significantly reduced using a Bap31 construct lacking the putative death effector homology domain (Bap31Δ167-240-Flag, lane 2). In fact, the residual amount of Ced-4 that was recovered in association with the Bap31 deletion mutant (lane 2) may have resulted because immunoprecipitation of Bap31Δ167-240-Flag with anti-Flag antibody also precipitated endogenous Bap31 (lanes 7). The latter is consistent with our findings that Bap31 forms homooligomers both *in vitro* and *in vivo* (unpublished). Of note, expression of Ced-4 did not lead to significant caspase-dependent cleavage of Bap31. This is concordant with the observation that Ced-4 does not independently induce apoptosis in 293T cells (9).

Importantly, the presence of Bcl-X_L did not influence the ability of Ced-4 to associate with Bap31 in 293T cells (Fig. 3A, lanes 4 and 6), suggesting that Bcl-X_L does not function to prevent Ced-4 from engaging the Bap31 complex. Moreover, this was also extended to procaspase-8, where it was found that Ced-4 and procaspase-8, when expressed individually in co-transfected 293T cells, associated with Bap31-Flag (Fig. 3B, lanes 3 and 5) to the same extent as in the situation where Ced-4 and procaspase-8 were expressed together (lane 7). Again, as was the case for Ced-4 (Fig. 3A), the ability of procaspase-8 to associate with Bap31 was significantly reduced by deleting the putative Bap31 death effector homology domain (Fig. 3C).

One obvious explanation to account for the ability of Ced-4 to associate with the Bap31 complex is that Ced-4 may be substituting for an endogenous Ced-4-like adaptor molecule. Earlier studies identified a means of testing this hypothesis (ref. 6, see schematic in Fig. 4). A mutant Ced-4 lacking the C-terminal 248 amino acids, Ced4Δc, failed to interact with Ced-9 or Bcl-X_L

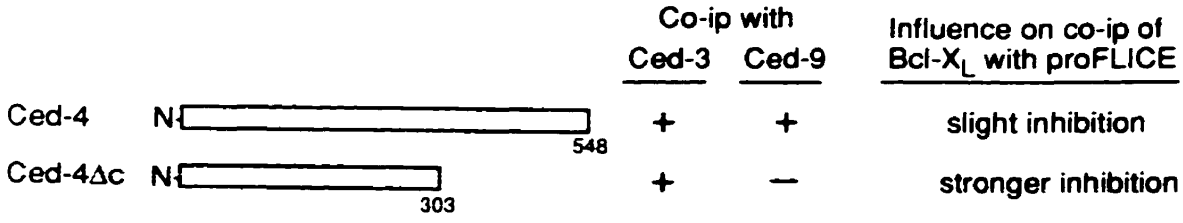
Figure 3. Ced-4 interacts with Bap31 in 293T cells. *A*, Ced-4 associates with the cytosolic domain of Bap31. 293T cells were transfected with the indicated vectors and, after 30 h, protein complexes were recovered from cell extracts (lysate) with anti-Flag antibody (Flag ip), resolved by SDS PAGE, transferred to nitrocellulose, and treated with either anti-HA antibody to detect Ced-4 (lanes 1-6) or chicken anti-Bap31 (α p28-M, ref. 20) (lanes 7-9) to detect Bap31 (Fig. 1). *B*, As in (A), except that protein complexes were recovered from cell extracts (lysate) with anti-Flag antibody (Flag ip), and Ced-4 and procaspase-8 (proFLICE) visualized with anti-HA antibody. *C*, As in (A) and (B), except that the association of procaspase-8 (proFLICE) with Bap31 and Bap31Δ167-240 was determined.

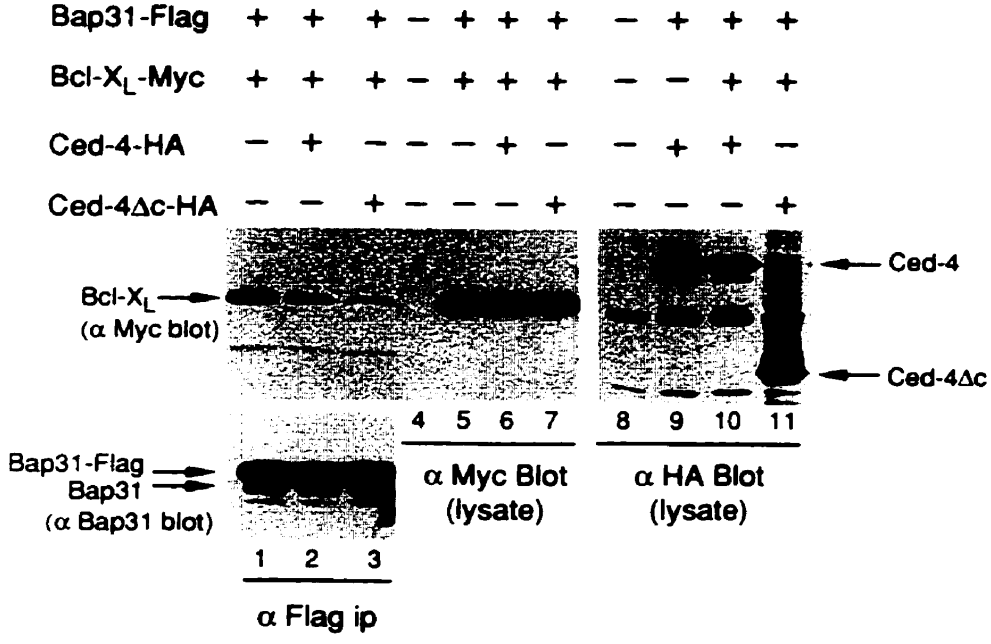

A
Control-Flag
Bap31-Flag
Bap31Δ167-240-Flag
Bcl-X$_L$-Myc
Ced-4-HA
Ced-4 (lysate)
Ced-4 (Flag ip)
Bap31-Flag
Bap31
Bap31 Δ167
240-Flag
1 2 3 4 5 6
α HA Blot
7 8 9
α Flag ip
α Bap31 Blot
B
Control-Flag
Bap31-Flag
Ced-4-HA
pro-FLICE-HA
Ced-4 (lysate)
pro-FLICE (lysate)
Ced-4 (Flag ip)
pro-FLICE (Flag ip)
1 2 3 4 5 6 7
α HA Blot
C
Control-Flag
Bap31-Flag
Bap31Δ167-240-Flag
pro-FLICE-HA
pro-Flice (lysate)
pro-Flice (Flag ip)
1 2 3 4
α HA Blot

but retained the ability to associate with Ced-3 or procaspase-8 in co-transfected 293T cells. Thus, it competes *in vivo* with an endogenous adaptor that bridges Bcl-X_L and procaspase-8 and prevents precipitation of procaspase-8 by an antibody directed to Bcl-X_L (6). Similarly, we found that Ced-4Δc lacking the C-terminal 246 amino acids also competed for the ability of Bcl-X_L to bind to Bap31 *in vivo* (Fig. 4, lanes 1 and 3, upper panel), strongly implying that it does so by displacing an endogenous adaptor which otherwise would make contact with Bcl-X_L. Wild-type Ced-4 also reduced somewhat the level of Bcl-X_L that was recovered with Bap31 (lanes 1 and 2), suggesting that Ced-4 is less efficient than the endogenous adaptor molecule at contributing to the association of Bcl-X_L with the Bap31 complex. Taken together, the results in Fig. 4 and those in Fig. 1B demonstrate that cooperative interactions between Bcl-X_L and both Ced-4 and procaspase-8 contribute to the stable binding of Bcl-X_L to Bap31 in vivo.

In conclusion, the ability of Ced-9 to bind to and prevent Ced-4 from activating Ced-3 provides a simple explanation to account for the molecular control over apoptotic cell death . Nevertheless, it remains to be explained how the corresponding molecular complexes in mammalian cells are linked to the multitude of signals that can trigger activation of procaspases, and how they interface with the numerous proapoptotic and antiapoptotic regulators that modulate these signals (24,29). The cooperative associations between Bcl-X_L, Ced-4, procaspase-8, and the Bap31 cytosolic domain observed here, on the other hand, suggest that Bap31 may provide one such bridge, and predict that an endogenous Ced-4-like adaptor molecule may be a normal constituent of the Bap31 complex.

Figure 4. Ced-4Δc displaces Bcl-X_L from the Bap31 complex. 293T cells were transfected with vectors encoding the indicated proteins and, 30 h later, cell extracts (lysate) were prepared, protein complexes isolated with anti-Flag antibody (α Flag ip), and the presence of Bcl-X_L (α Myc), Ced-4 or Ced-4Δc (α HA), and Bap31 (α Bap31 using the p28-M antibody) assessed following SDS PAGE and immunoblotting, as described in Figs. 1 and 3. See text for a description of the schematic above the figure.


Co-ip with
Ced-3
Ced-9
Influence on co-ip of Bcl-X$_L$ with proFLICE
Ced-4
N
548
+
+
slight inhibition
Ced-4Δc
N
303
+
—
stronger inhibition


Bap31-Flag + + + — + + + — + + +
Bcl-X$_L$-Myc + + + — + + + — — + +
Ced-4-HA — + — — — + — — + + —
Ced-4Δc-HA — — + — — — + — — — +
Bcl-X$_L$
(α Myc blot)
Ced-4
Ced-4Δc
4 5 6 7
8 9 10 11
α Myc Blot
(lysate)
α HA Blot
(lysate)
Bap31-Flag
Bap31
(α Bap31 blot)
1 2 3
α Flag ip

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[1]The abbreviations used are: ER, endoplasmic reticulum; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Connecting text

In the proceeding chapter, we further characterized p28Bap31 by showing that it resided primarily in the endoplasmic reticulum and possessed the ability to form homodimers, and possibly oligomers, *in vivo*.

Chapter IV

p28Bap31 is an Endoplasmic Reticulum membrane protein which forms Homodimers/-oligomers *in vivo*

Summary

Bcl-2 and its related proteins are cardinal for the regulation of apoptosis in mammalian cells. Despite much effort, the mechanism of action of the Bcl-2 family members remains unclear. Recently we identified p28Bap31 as a Bcl-2 interacting protein. *In vitro* targeting experiments showed that p28Bap31 can be inserted into endoplasmic reticulum microsomes in an N_{lumen}-C_{cyto} orientation exposing the carboxy-half of the protein to the cytoplasm (Ng et al., 1997). By biochemical fractionation and immunogold labeling, it was confirmed that p28Bap31 is localized to the endoplasmic reticulum membrane. Moreover, chemical crosslinking and co-immunoprecipitation experiments revealed that p28Bap31 may exist as both homodimers and oligomers *in vivo*.

Introduction:

Apoptotic programmed cell death is an intrinsic cellular suicide pathway which allows the rapid elimination of unwanted or harmful cells. It is essential for embryogenesis, homeostasis and host defense against viral infection (Kerr et al., 1972; Reed, 1994; Saunders, 1966). Members of the Bcl-2 family have been shown to play an important role in regulating apoptosis (Kroemer, 1997; Reed, 1994). All of these proteins, with the exception of Bad (Yang et al., 1995) and Bid (Wang et al., 1996) which share the least sequence homology with Bcl-2, contain carboxy-terminal hydrophobic transmembrane domains which are responsible for the targeting to intracellular membrane sites (Kroemer, 1997). The anti-apoptotic protein Bcl-2 has been localized to the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope (Akao et al., 1994; Chen-Levy et al., 1989; Monaghan et al., 1992; Nguyen et al., 1993). Deletion of its carboxy signal anchor sequence not only abolishes its ability to associate with cytoplasmic membranes, it also impairs its ability to protect cells against apoptosis (Nguyen et al., 1994). Replacement of the transmembrane sequence of Bcl-2 with a homologous signal anchor sequence found in yeast outer mitochondrial protein, Mas70, which also targets the chimeric protein to the correct location and orientation, restores its anti-apoptotic function (Nguyen et al., 1994). This suggests that the correct localization and orientation of the protein, but not the actual transmembrane sequence, contribute to its function. It also raises the possibility that Bcl-2 may function to suppress cell death by regulating events which occur at the intracellular membrane sites where it is located.

Recently, the role of the mitochondrial location of Bcl-2 with respect to its protective effect has been addressed. It has been demonstrated that

cytochrome c and AIF (apoptosis-inducing factor) are released from the mitochondria into the cytosol where they trigger the events that lead to the activation of Caspase-3 (Kluck et al., 1997; Susin et al., 1996; Yang et al., 1997). Expression of Bcl-2 blocks their translocation and the processing of the protease. Moreover, the addition of exogenous cytochrome c bypass the protective effect of Bcl-2 (Kluck et al., 1997), indicating that the function of Bcl-2 in mitochondria may be to prevent the factors which are required for the activation of the caspases from translocating into the cytosol.

To date, the biochemical mechanism by which cytochrome c and AIF are translocated into the cytosol is unclear. Nevertheless, it has been proposed that the opening of the permeability transition (PT) pore (Kroemer et al., 1997; Zamzami et al., 1995; Zamzami et al., 1995), stimulated by apoptotic signals, causes the collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and subsequent release of apoptogenic macromolecules. Porin (a voltage-dependent anion channel), cardiolipin synthase, phospholipid hydroperoxide glutathione peroxidase have all been suggested to be components of the PT-pore (Kroemer et al., 1997). However, the precise identity of the molecules which constitute PT pore is thus far elusive. Recently several *in vitro* studies showed that Bcl-2, Bcl-X_L and Bax all possess channel/pore forming properties in synthetic lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). Combined with the fact that expression of Bcl-2 or Bcl-X_L inhibit the collapse of $\Delta\Psi_m$ and PT pore formation, it is conceivable that these proteins may regulate the opening and closing of the PT-pore. The channel forming properties of these proteins *in vivo* has yet to be determined.

Despite the current focus on mitochondria, Bcl-2's localization to the ER is likely also important for its function. A recent study showed that

redirection of Bcl-2 to specific organelles limited the ability of the chimeric proteins to inhibit apoptosis in different cell types (Zhu et al., 1996), indicating that the existence of Bcl-2 in various intracellular membranes may ensure maximum protection against programmed cell death in response to various apoptotic stimuli. For example, Bcl-2 may function to recruit interacting proteins to multiple organelles, or Bcl-2 may associate with specific components of these membranes in order to regulate apoptosis. It is also conceivable that during apoptosis signals are shuttled between the organelles, and the presence of Bcl-2 provides multiple sites or levels of regulation.

Recently, we reported the identification of p28Bap31 as a Bcl-2/Bcl-X_L interacting protein (Ng et al., 1997). It contains three predicted transmembrane domains at its amino-terminus and a potential death effector domain (Chinnaiyan et al., 1995) overlapping with a predicted leucine zipper, both of which are flanked by identical caspase cleavage sites. It also has a canonical ER-retrieval signal at its extreme carboxy-terminus (Jackson et al., 1993).

In addition to Bcl-2 and Bcl-X_L, p28Bap31 also interact with both procaspase-8 (Ng et al., 1997) and Ced-4 in co-transfected 293T cells (Ng et al., 1998). This raises the possibility that the complex formed between p28Bap31, Bcl-2/Bcl-X_L, procaspase-8 and a mammalian Ced-4-like-molecule is an integral component of the cell death pathway. In order to gain further insight of the role of p28Bap31 in apoptosis, its subcellular localization and topography *in vivo* were investigated for this report.

Materials and methods:

Generation of p28(165-246) and HA-tagged p28(165-246): The cDNA fragment corresponding to p28 amino acids 165-246 was generated by PCR using GGCCGGATCCAGAATGGTGACTCTCATTTCG CAGC as sense oligo and GCGAATTCTTACTCTTCCTTCTTGTC as anti-sense oligo. HA-tagged p28 (165-246) was generated by PCR using GGCCGGATCCACC ATGGCGTACCCATACGATGTTCCAGATTACGCTGTGACTCTCAT TTCGCAGC as sense oligo and GCGAATTCTTACTCTTCCTTCTTGTC as anti-sense oligo. The resulting fragments were cloned into BamH I - EcoR I site of pBluescript KS plasmids (Promega). Authenticity was confirmed by nucleotide sequencing.

Coimmunoprecipitations: The ^{35}S-labeled proteins generated by *in vitro* transcription-translation in rabbit reticulocyte lysate was incubated with 15μg of anti-HA monoclonal antibody in 200μl of binding buffer (10mM Hepes, pH 7.2, 140mM KCl, 5mM $MgCl_2$, 1mM EGTA, 0.05% NP40). The precipitates were recovered and washed extensively on protein A sepharose (Pharmacia). The associated translated proteins were visualized by SDS-PAGE and fluorography.

293T cells in 10-cm culture plates were transfected with 10μg of total DNA when cells reached 50-60% confluency. The coimmunoprecipitation was performed as described previously (Ng et al., 1997).

Cross-linking of p28Bap31; A non-reversible, homobifunctional cross-linking agent BS^3 was used and the detail was as described in figure 4.

Preparation of cellular and subcellular membrane fractions: Various membrane fractions were prepared from rat hepatocytes. See Paiement and Bergeron (1983) for detail procedures.

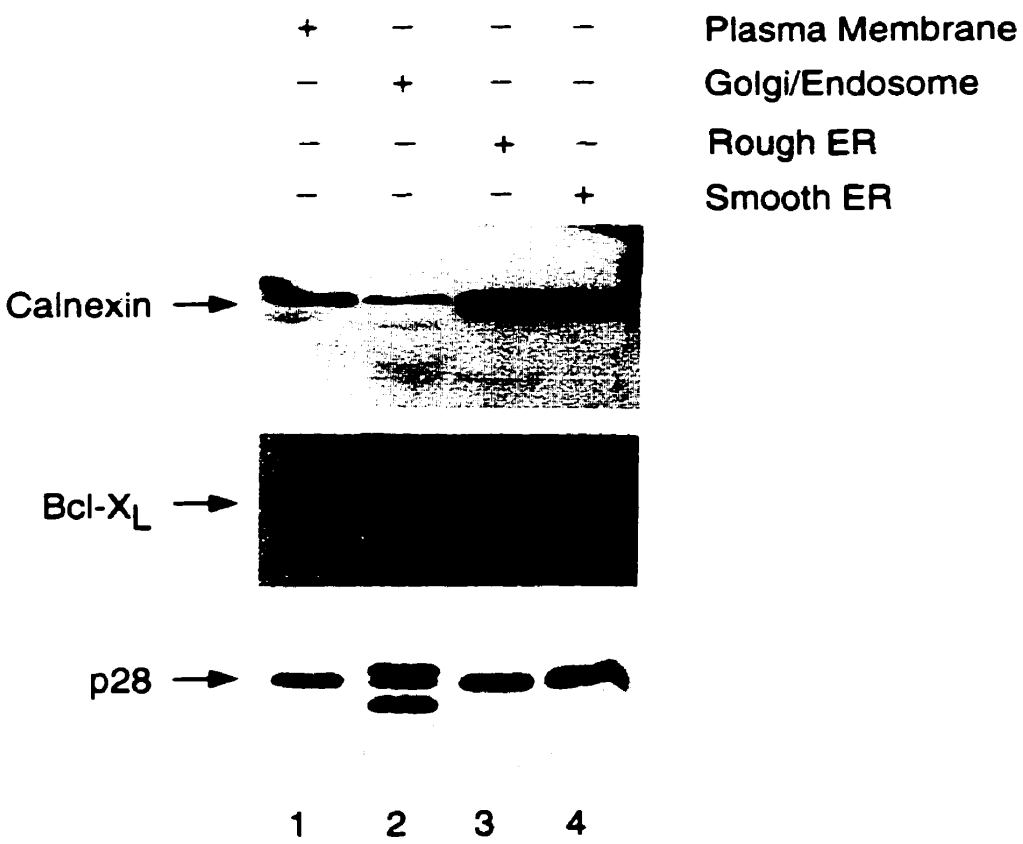
Electron microscopy: Tissue preparation for cryo-immune EM was carried our as described previously (Dahan et al., 1994).

Results and Discussion:

The presence of the ER retrieval signal (Jackson et al., 1993) at the extreme carboxyl-terminus of p28Bap31 suggests that it may be an ER resident. We have shown previously that p28Bap31 can be co-translationally inserted into purified ER microsomes in an N_{lumen}-C_{out} orientation leaving the c-terminal half of the protein facing the cytoplasm (Ng et al., 1997). To further characterize the ultrastructural localization of p28Bap31, an initial step was to determine the expression level of the protein in various biochemically fractionated membrane fractions. These fractions included the plasma membrane (PM), Golgi, rough ER and smooth ER purified from rat liver hepatocytes. When equal amounts of protein from each fraction were analyzed by immunoblotting using p28Bap31 specific antibody, αp28M (Ng et al., 1997), the ER fractions showed a higher p28Bap31 expression level than the other membrane fractions examined (Fig. 1, bottom panel). In cells, the ER membrane has a surface area at least 50-fold larger than either the plasma membrane and Golgi apparatus, respectively. Therefore, there is a substantial difference in the total amount of p28Bap31 proteins in the ER fractions relative to the others. Of note, three bands were detected by αp28M antibody in the Golgi apparatus, indicating that p28Bap31 may be processed within this compartment. In agreement with previous findings, Bcl-X_L was localized to the ER membrane, thus displaying a similar distribution pattern as that of p28Bap31 (Fig. 1, middle panel). The co-expression of the two proteins in the ER membrane further supports the idea that the interaction of these two proteins *in vivo* may occur at this intracellular site. In order to monitor the purity of the membrane fractions, the same sample were analyzed by immunoblotting using antibody against calnexin, a well-characterized ER

Figure 1: Subcellular distribution of p28Bap31.

The subcellular distribution of p28Bap31 was analyzed by immunoblotting on four different biochemically fractionated membrane fractions. Using a p28Bap31 specific antibody (Ng et al., 1997), it was shown that p28Bap31 was enriched in the ER fractions (bottom panel, lanes 3 and 4) and it showed similar distribution pattern as Bcl-X_L (middle panel). An ER specific protein, calnexin, was used as a control (top panel).


+ – – – Plasma Membrane
– + – – Golgi/Endosome
– – + – Rough ER
– – – + Smooth ER
Calnexin
Bcl-X$_L$
p28
1 2 3 4

membrane protein (Wada et al., 1991). As expected calnexin was detected almost exclusively in the ER fractions (Fig. 1, top panel).

The ER localization of p28Bap31 *in vivo* was further confirmed by immuno-electron microscopy of rat hepatocytes using an anti-p28 (122-164) rabbit polyclonal antibodies. p28Bap31 protein was found to be highly associated with the perinuclear ER membrane (Fig. 2A-D). The immunogold labeling of the protein was quantitated and summarized in Table 1. In summary, over 75 per cent of the p28Bap31 protein was associated with the ER, both smooth and rough ER, while a small amount was distributed among the plasma membrane, mitochondria and Golgi apparatus. In contrast, pre-immune sera failed to produce membrane associated gold-labeling (Fig. 2B).

As mentioned earlier, p28Bap31 also contains a putative leucine zipper at its carboxyl terminus. Leucine zippers are shown to mediate protein-protein interaction and are commonly employed by transcription factors and cellular proteins for dimerization (Neuberg et al., 1989; Sassone-Corsi et al., 1988; Struhl, 1989). To determined whether p28Bap31 can homodimerize, we looked at the ability of p28Bap31 to co-precipitate with itself in *in vitro*. p28(165-246), which lacks the transmembrane domains, was translated alone or with a HA-tagged p28(165-246) (see schematics in Fig. 3A, bottom panel). In the absence of HA-p28(165-246), there was no precipitation of p28(165-246) by anti-HA monoclonal antibody (Babco) (Fig. 3A, lane 4). However, the non-tagged protein was precipitated in the presence of HA-p28(165-246) (Fig. 3B, lanes 6), showing an association between the two versions of p28(165-246).

We then determined if this self-association occurs *in vivo*. An antibody to the FLAG epitope (M2, Kodak) immunoprecipitated endogenous

Figure 2: p28Bap31 is an ER resident.

The ultrastructural localization of p28Bap31 was determined in rat cells by immunogold labeling under electron microscope. **A-D.** Rat liver hepatocytes were prepared for cryo-immune EM as described by Dahan et al., 1994. The cells were incubated with anti-human p28Bap31 rabbit antibody αp28(122-164) followed by gold-conjugated anti-rabbit IgG. The staining of the gold particles (indicated by the arrows), which was highly associated with the ER membrane, was viewed under electron microscope. **E.** Labeling was not observed with pre-immune serum. SeeTable 1 for quantitation.

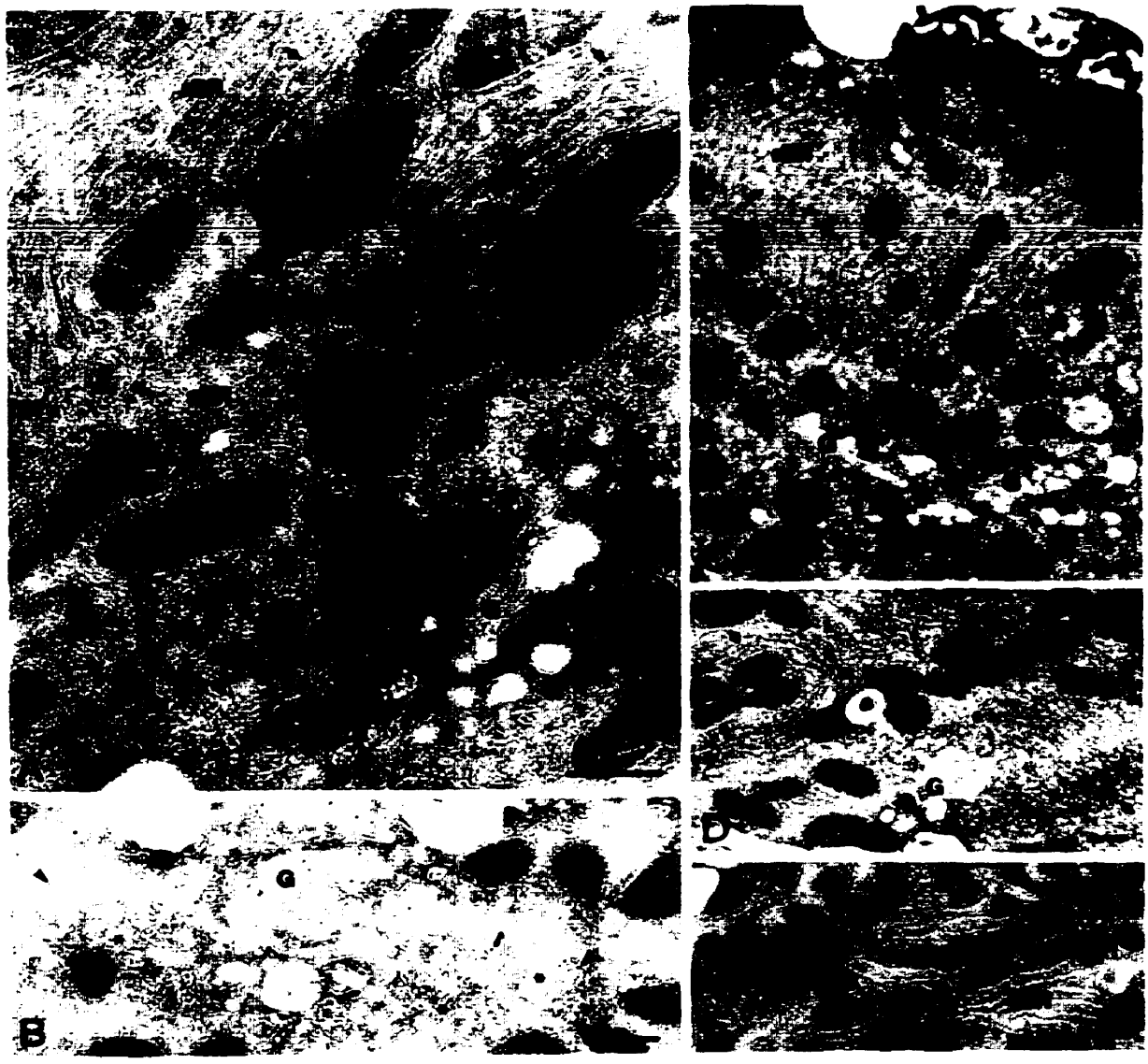

B
C
D

Table 1. Intracellular distribution of p28Bap31 in rat liver hepatocytes[1]

Cell Compartment	No. Particles Scored[2]	% Distribution
Rough ER	2009	21.7
Smooth ER	5081	55.0
Golgi Apparatus	193	1.7
Plasma Membrane[3]	856	9.2
Mitochondria	800	8.6
Peroxisomes	167	1.8
Lysosomes	25	0.3
Multivesicular Endosomes	111	1.2

1 Data from rat liver hepatocyte cryosections from 4 animals.

2 Liver hepatocytes with well preserved morphology were photographed at a magnification of 9000X. Gold particles were allocated to the compartments indicated. A cytoplasmic profile area of approximately 2530μm^2 (i.e., excludes nuclear profile areas) was evaluated for gold particles labeling in sections of 19 hepatocytes that were labeled for p28Bap31, and sections of 20 hepatocytes that were labeled with preimmune serum. Gold particle counts over each structure were adjusted for gold particule counts over an equivalent area of the structure stained with preimmune serum.

3 Includes sinusoidal and bile canalicular plasma membrane surfaces.

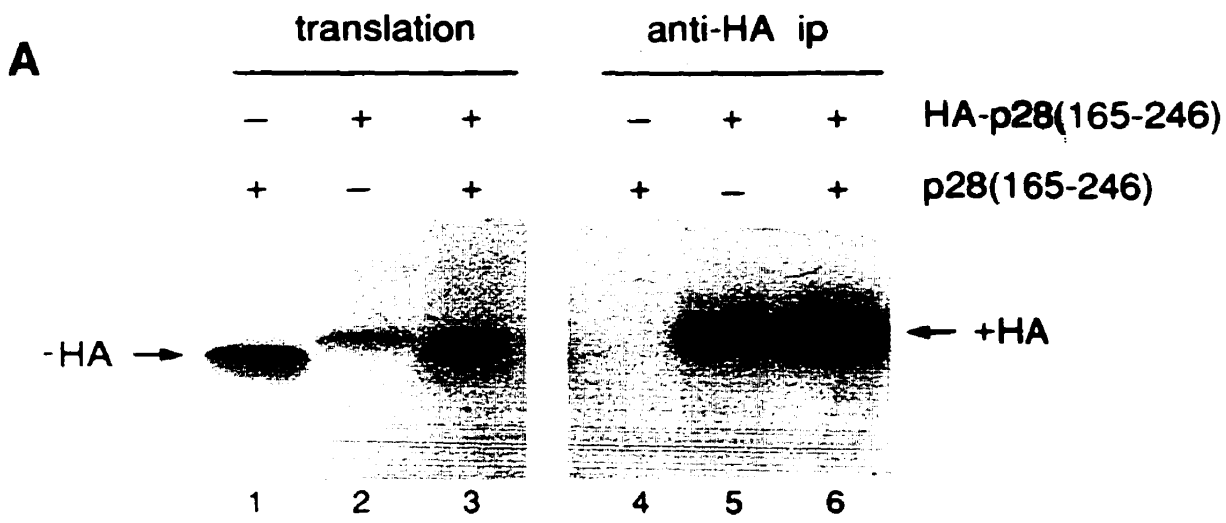
p28Bap31 from 293T cells transfected with FLAG-tagged p28 (Fig. 3B, lane 3), but not from cells transfected with the control FLAG vector (Fig. 3B, lane 2). The ability of FLAG-p28 to precipitate endogenous p28Bap31 confirms the result observed *in vitro* and supports the existence of p28Bap31 oligomers *in vivo*. Interestingly when FLAG-p28(165-246) was transfected into 293T, the amount of endogenous p28Bap31 precipitated decreased dramatically (data not shown), implying that homodimerization of p28Bap31 is dependent not solely on the leucine zipper, but also on other regions of the protein.

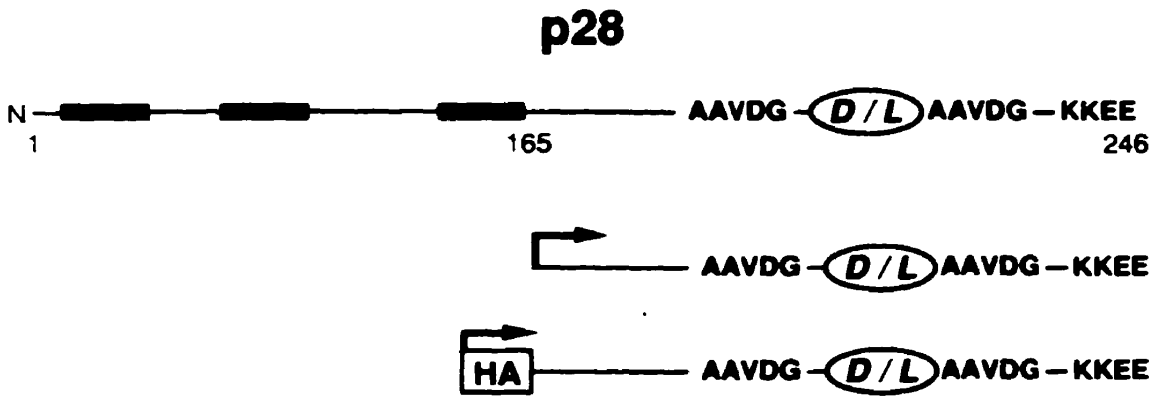
Moreover, when purified ER was incubated with a homobifunctional cross-linking agent, bis(sulfo-succinimidyl) suberate, followed by immunoblotting using αp28M, a band near the predicted size of the homodimeric form of p28Bap31 was observed (Fig. 4, marked by the arrow). Interestingly, a band which showed a slightly slower mobility than the apparent p28Bap31 homodimer was also observed (marked by the asterisk, Fig. 4). It may indicate a trimer or another yet-to-be identified ER protein which is associated with p28Bap31. Despite the fact that multimeric forms of p28Bap31 were not clearly demonstrated in this experiment, preliminary result obtained from gel filtration experiments by FPLC showed that endogenous p28Bap31 indeed form higher order structures (unpublished data). The ability for p28Bap31 to form dimers and higher order structures containing Bcl-2/Bcl-X_L, Ced-4-like molecule and procaspase-8 indicates the stability of such structures may be involved in the regulation of cell death. As shown in a previous study, overexpression of the 20kDa caspase-cleavage product of p28Bap31 induces cell death (Ng et al., 1997). The mechanism by which it triggers cell death may be by acting as a dominant

Figure 3: Dimerization of p28Bap31 both *in vitro* and *in vivo*.

A. p28(165-246), lacking the three predicted transmembrane domains, was co-translated with HA-tagged p28(165-246) *in vitro* in rabbit reticulocyte lysate in the presence of ^{35}S-Met. The labeled lysate was precipitated with anti-HA monoclonal antibody (Babco) and the precipitates were analyzed by SDS-PAGE and autoradiography. The positions of p28(165-246) and HA-p28(165-246) were indicated by the arrows. Non-tagged p28(165-246) was precipitated along with HA-tagged p28(165-246) (lane 6). As controls, *in vitro* translated p28(165-246) or HA-tagged p28(165-246) alone was incubated with anti-HA monoclonal antibody. As expected HA-tagged p28(165-246) was precipitated by the antibody (lane 5) while the anti-HA monoclonal antibody failed to bring down the non-tagged p28(165-246) (lane 4). The 10% input of each of the translation product was shown in lanes 1-3.

B. Epitode tagged p28Bap31 can precipitate endogenous p28Bap31 *in vivo*. FLAG-tagged p28Bap31 was transfected into 293T cells. 30 hours post-transfection the total cell lysate was precipitated with anti-FLAG monoclonal antibody (Kodak). The precipitates were analyzed by immunoblotting using αp28-M chicken antibody. As shown in lane 3 endogeneous p28Bap31 co-precipitated with the FLAG-tagged p28Bap31 while control FLAG showed no precipitation (lane 2). Endogenous p28 and transfected FLAG-tagged p28 were indicated by the arrows.

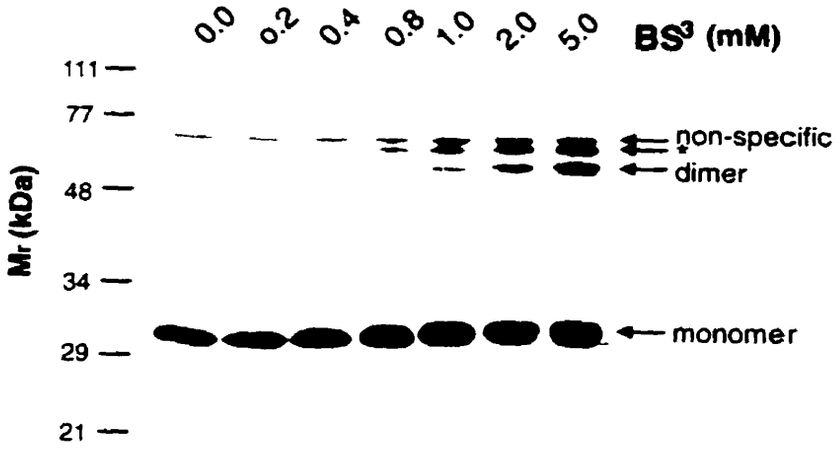

A
translation
anti-HA ip
HA-p28(165-246)
p28(165-246)
-HA
+HA
1
2
3
4
5
6


p28
N
1
165
AAVDG
D/L
AAVDG—KKEE
246
HA


B
Total cell lysate
FLAG vector
p28Bap31/FLAG
p28Bap31/FLAG
p28Bap31 (endogenous)

Figure 4: Cross-linking of endogenous p28Bap31.

150μg of purified total ER proteins were incubated with different concentrations of bis(sulfosuccinimidyl) suberate (BS^3) in 150μl cross-linking buffer (20mM Hepes at pH 7.4, 75mM KCl, 1mM MgCl2, 1mM EDTA and 10% v/v glycerol) at room temperature for 30 min. The samples were anzlyzed by SDS-PAGE followed by immunoblotting using αp28-M antibody. The positions of p28 monomers, dimers and a non-specific cross reacting protein were indicated by the arrows. A band with a slightly slower mobility than the p28Bap31 dimer was also detected. It was denoted by an *asterisk*.


0.0
0.2
0.4
0.8
1.0
2.0
5.0
BS³ (mM)
111
77
48
34
29
21
Mr (kDa)
non-specific
dimer
monomer

negative of p28Bap31 which causes the disassembly of the complex. However, this remains to be determined.

Several studies have shown that mitochondrial Bcl-2/Bcl-X_L proteins prevent the release of factors that stimulate the activation of caspase-3. However, the role of the ER in this process remains unclear. The identification of p28Bap31 may provide clues on how Bcl-2/Bcl-X_L proteins function at the ER. p28Bap31 is the first ER protein which conduits an indirect link between Bcl-2 and procaspase-8 (Ng et al., 1997). Procaspase-8 has been shown to be recruited to the Fas/TNFR1 signaling complexes and functions as an apex caspase which triggers the activation of downstream family members. Nevertheless, Fas/TNFR1 responds to only a limited set of apoptotic stimuli. Therefore, the p28Bap31 complex in the ER likely provides an intracellular membrane site for the recruitment of procaspase-8 upon induction of apoptosis by other stimuli. The presence of Bcl-2/Bcl-X_L in this complex may be to suppress the activation of initiator caspases like caspase-8. It is conceivable that apoptotic signals oscillate between mitochondria and the ER and trigger the activation of both initiator and effector caspases. The presence of Bcl-2/Bcl-X_L at both membrane sites ensures maximum protection against cell death.

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Chapter V
Discussion

Discussion:

Programmed Cell death (apoptosis) has emerged as a fundamental cellular process in multicellular organisms. Proper regulation of this genetically scheduled suicide program is essential for both embryogenesis and homeostasis. When it is turned on or off unexpectedly due to genetic mutations, external stimuli or viral infection, the consequence can be harmful. In some cases it leads to detrimental disease states, such as cancer, neurodegenerative diseases and AIDS. The ability to manipulate apoptosis represents a novel approach for disease treatment. For instance, the elimination of tumour cells can be achieved by specific activation of the effector molecules which are responsible for the execution of apoptosis. On the other hand, neurodegenerative propensity can be controlled by inhibiting aberrant apoptotic cell death through the activation of survival factors. Therefore, a complete understanding of how apoptotic programmed cell death is regulated *in vivo* is cardinal in order to exploit its therapeutic potential.

Although much progress has been made in the past decade, the core apoptotic signaling pathway has not been fully elucidated. One of the major players of PCD in mammalian cells is the Bcl-2 proto-oncoprotein (Cleary et al., 1986; Hockenbery et al., 1990). Bcl-2 and its related proteins constitute a PCD regulatory family within which there are both apoptosis antagonists (Bcl-2, Bcl-X_L, and Bcl-w) (Boise et al., 1993; Gibson et al., 1996; Hockenbery et al., 1990) and agonists (Bax, Bak, Bad, Bid, and Bik) (Boyd et al., 1995; Farrow et al., 1995; Oltvai et al., 1993; Wang et al., 1996; Yang et al., 1995). Bcl-2 functions to block apoptosis mediated by a wide range of stimuli (Reed, 1994). Transgenic animal models indicate that Bcl-2 actively participates in the regulation of PCD (McDonnell et al., 1989; McDonnell and Korsmeyer,

1991), and thus it is of great interest to identify its interacting proteins. This thesis describes the identification and characterization of a Bcl-2 interacting protein, p28Bap31.

In chapter two, the identification and characterization of p28Bap31 was described. Using labeled Bcl-2 as a probe in a Far Western analysis (Blanar and Rutter, 1992), a protein with apparent molecular weight of 20kDa (p20) was identified. The appearance of this protein was induced during apoptosis elicited by adenovirus type5 mutants which lack functional E1B 19k (Debbas and White, 1993). p20 was purified in series of biochemical fractionations based on its physical properties. The final purified product was subjected to amino acid sequencing by Edman N-terminal degradation. The partial sequence obtained belonged to a 28kDa protein, Bap31 (p28Bap31). When co-expressed in 293T cells, p28Bap31 could interact with both Bcl-2 and Bcl-X_L *in vivo*, but not with Bax. Interestingly, the co-expression of Bax could prevent Bcl-2 from interacting with p28Bap31, presumably by acting as a competitor for interaction with Bcl-2. p28Bap31 was previously identified as a B-cell receptor associating protein (Bap) which was brought down in a complex with the B-cell receptor in co-immunoprecipitation experiments (Adachi et al., 1996). This protein contains several interesting features. It possesses three predicted transmembrane domains at its amino-terminus, a putative cytosolic leucine zipper and a canonical ER retrieval signal (KKXX, using one letter amino acid code) (Jackson et al., 1993) at its extreme carboxy-terminus. Finally amino acid sequence alignment also revealed that p28Bap31 contains a putative death effector domain (DED) which overlaps with the leucine zipper and is flanked by identical caspase cleavage sites at positions 164 and 238.

Since the DEDs found in the Fas/TNFR1 signaling complexes are responsible for the recruitment of procaspase-8 (Muzio et al., 1996), we wished to determine whether procaspase-8 could also be recruited to p28Bap31 in the presence or absence of Bcl-X_L. In co-transfected 293T cells, p28Bap31 could simultaneously associate with both Bcl-X_L and procaspase-8. This suggests that p28Bap31, Bcl-2/Bcl-X_L and procaspase-8 can form at least a ternary complex *in vivo*.

In order to determine whether p28Bap31 was cleaved at the predicted caspase cleavage sites, *in vitro* translated p28Bap31 was incubated with various purified caspases. It was demonstrated that initiator Caspase-8 showed the highest proteolytic activity toward p28Bap31, when compared with Caspase-1 and Caspase-3 (Chapter 2, Fig. 5), yielding two cleavage products corresponding to cleavage sites at positions 164 and 238. Using a p28Bap31 specific antibody, this cleavage was also assessed *in vivo* during apoptosis. The result indicated that the same cleavage occurred during apoptosis giving rise to a similar cleavage pattern. Then we looked at the effect of Bcl-2 on the cleavage of p28bap31. In the absence of Bcl-2, p28Bap31 was cleaved during apoptosis induced by the expression of E1A. However, when cells overexpressed Bcl-2, the proteolytic cleavage of p28Bap31 as well as apoptosis were inhibited. We then addressed whether ectopic expression of either p28Bap31 or p20 was toxic to cells. Each protein was transiently expressed in human 293T cells along with a luciferase reporter gene in order to measure the cytotoxicity of each polypeptide. Ectopic expression of the p20, but not the full length p28Bap31, suppressed the transcription activity of the reporter gene by inducing apoptosis which can be overcome by co-expression of Bcl-2 .

In *C. elegans*, Ced-4 can mechanically link Ced-3 to Ced-9 by interacting simultaneously with both proteins (Chinnaiyan et al., 1997). Similar observation was extended to mammalian system. When Ced-4 was co-transfected into human 293T cells, it could also link Bcl-2 to procaspase-8, the mammalian counterparts of Ced-9 and Ced-3 respectively (Chinnaiyan et al., 1997). Based on these studies and the fact that direct interaction between p28Bap31 and procaspase-8 was not detected by *in vitro* binding experiments, it is possible that the association between these proteins is mediated by a Ced-4-like adaptor molecule.

Chapter three describes our attempts to determine whether a Ced-4-like molecule is part of the p28Bap31 complex. *In vitro* Ced-4 interacted with a GST-fusion protein containing the cytosolic region of p28Bap31 (aa#120-246), presumably through DED-DED ineraction, but not with GST alone. This association was confirmed *in vivo* by co-immunoprecipitation. Moreover, in co-transfected 293T cells, the interaction between Ced-4 and p28Bap31 was not affected by the expression of Bcl-X_L or procaspase-8, rather, these proteins associate with p28Bap31 in a cooperative manner. This suggests that a mammalian Ced-4-like molecule is a normal constituent of the p28Bap31 complex. Furthermore, the association of these core apoptotic regulatory proteins to p28Bap31 supports the possibility that the complex formed by these proteins may play a role in the regulation of apoptosis.

In order to further characterize p28Bap31, we also investigated the subcellular localization and the topography of the protein. The presence of the transmembrane domains and the ER retention signal suggested that p28Bap31 may be an ER membrane protein, although previous study indicated that it associated with cell surface bound B-cell receptors (Adachi

et al., 1996). *In vitro* targeting to purified ER microsomes showed that p28Bap31 was inserted into the ER membrane in an N_{lumen}-C_{cyto} orientation, leaving the carboxy-terminal half of the protein facing the cytosol (Chapter 1, Fig. 3). In chapter four, the results obtained from biochemical fractionation and immunogold labeling experiments further confirmed that the majority of the p28Bap31 protein was found at the ER membrane. Not only does this result show that p28Bap31 is localized to the same subcellular site as Bcl-2, it also indicates the existence of the first Bcl-2 apoptotic regulatory complex in the ER membrane.

Several studies have attempted to address the mechanism of action of Bcl-2 with respect to its localization in the outer mitochondrial membrane. One of the early events during apoptosis is the loss of membrane potential in the mitochondria. It has been suggested that this is due to the opening of permeability transition (PT) megapores, which allows the uncensored flow of ions across the membrane and disruption of the electral chemical potential (Kroemer et al., 1997). Moreover, after the opening of such PT-pores, the escape of macromolecules from the inner membrane space also becomes possible. Cytochrome c and AIF have been suggested to be the critical macromolecules which are released during apoptosis; they, in turn, contribute to the activation of downstream caspases (Kluck et al., 1997a,b; Liu et al., 1996; Susin et al., 1996; Yang et al., 1997). It is found that the presence of Bcl-2 in the mitochondria inhibits the formation of the PT-pores and prevents cytochrome c and AIF from engaging/activating the cell death effector machinery/complex waiting in the cytosol (Kluck et al., 1997a; Susin et al., 1996; Yang et al., 1997). Therefore, from this point of view, the localization of Bcl-2/Bcl-X_L will be important for its ability to block

apoptosis. In fact, deletion of the signal anchor sequence of Bcl-2 has been shown to impair its anti-apoptotic function (Nguyen et al., 1994).

Unlike mitochondria, the role of the ER localization of Bcl-2 with respect to its anti-apoptotic function remains unclear and understudied. It is well documented that ER is a major source of intracellular Ca^{2+} and numerous Ca^{2+} receptors and pumps are found in the ER membrane in order to monitor Ca^{2+} level within the cells. Bcl-2 has been proposed to prevent apoptosis by regulating Ca^{2+} efflux at the ER level (Lam et al., 1994), albeit some studies show contradictions arguing that apoptosis is independent of the intracellular calcium ion level (Reynolds and Eastman, 1996). Recently, it was shown that ablation of the inositol 1,4,5-trisphosphate (IP_3) receptor-1, a calcium release channel residing in the ER membrane, confers resistance to various apoptotic signals suggesting that elevation of intracellular Ca^{2+} mediated by the IP_3 receptor-1 is critical for apoptosis (Jayaraman and Marks, 1997). It is plausible that the function of p28Bap31 complex at the endoplasmic reticulum may be related to the regulation of Ca^{2+} efflux by IP_3-receptors either directly or indirectly.

Finally, the ability of p28Bap31 to form homodimers or oligomers *in vivo* was also determined in chapter four. The presence of the leucine zipper, which has been shown to promote protein-protein interactions, indicated p28Bap31 may form homodimers or oligomers. *In vitro* co-immunoprecipitation experiments showed that epitode tagged-p28Bap31 could bring down the non-tagged version protein. In addition, endogenous p28Bap31 could be co-precipitated with the transiently transfected tagged proteins showing the ability for p28Bap31 to self-associate. Consistent with this, when endogenous p28Bap31 was chemically crosslinked, it yielded homodimeric species. Although the oligomeric forms of the protein were

not observed using this technique, it was shown that endogenous p28Bap31 could form higher order structures, which could be p28Bap31 oligomers or a complex form with other proteins, as determined by gel filtration experiments by FPLC (data not shown). Interestingly, deletion mutants of p28Bap31 which lacked the leucine zipper region did not abolish its ability to precipitate the endogenous proteins (data not shown), suggesting that p28Bap31 homodimerizes not only through its leucine zipper domain, but also through its transmembrane region.

Taken together, these results suggest that the p28Bap31 complex at the ER may be analogous to the Fas/TNFR1 complexes found at the plasma membrane. Since most of the apoptotic stimuli elicit cell death independent of the Fas/TNFR1, the recruitment of procaspase-8 to the ER by the p28Bap31 complex may represent an intracellular membrane site where procaspase-8 engages in the initiation of the caspase cascade. Therefore we propose the following model. Upon the induction of apoptosis, in the absence of Bcl-2/Bcl-X_L, caspase-8 is activated and dissociates from the p28Bap31/Ced-4/procaspase-8 ER complex. The activated caspase-8/caspase-8-related proteases in turn trigger the activation of downstream caspases, presumably at the mitochondrial level, such as caspase-3 which cleaves specific cellular targets to ensue irreversible destruction of the cellular network. The cleavage of p28Bap31 to p20, presumably by caspase-8-like proteases, may function to amplify the caspase cascade or simply activate a parallel death pathway. The integrity of this ER complex can be preserved by interaction with Bcl-2/Bcl-X_L. This interaction may cause conformational changes in p28Bap31 which prohibit the dissociation of procaspase-8. Moreover, since it has been shown that the presence of Bcl-2 in the outer mitochondrial membrane prevents the release of cytochrome c

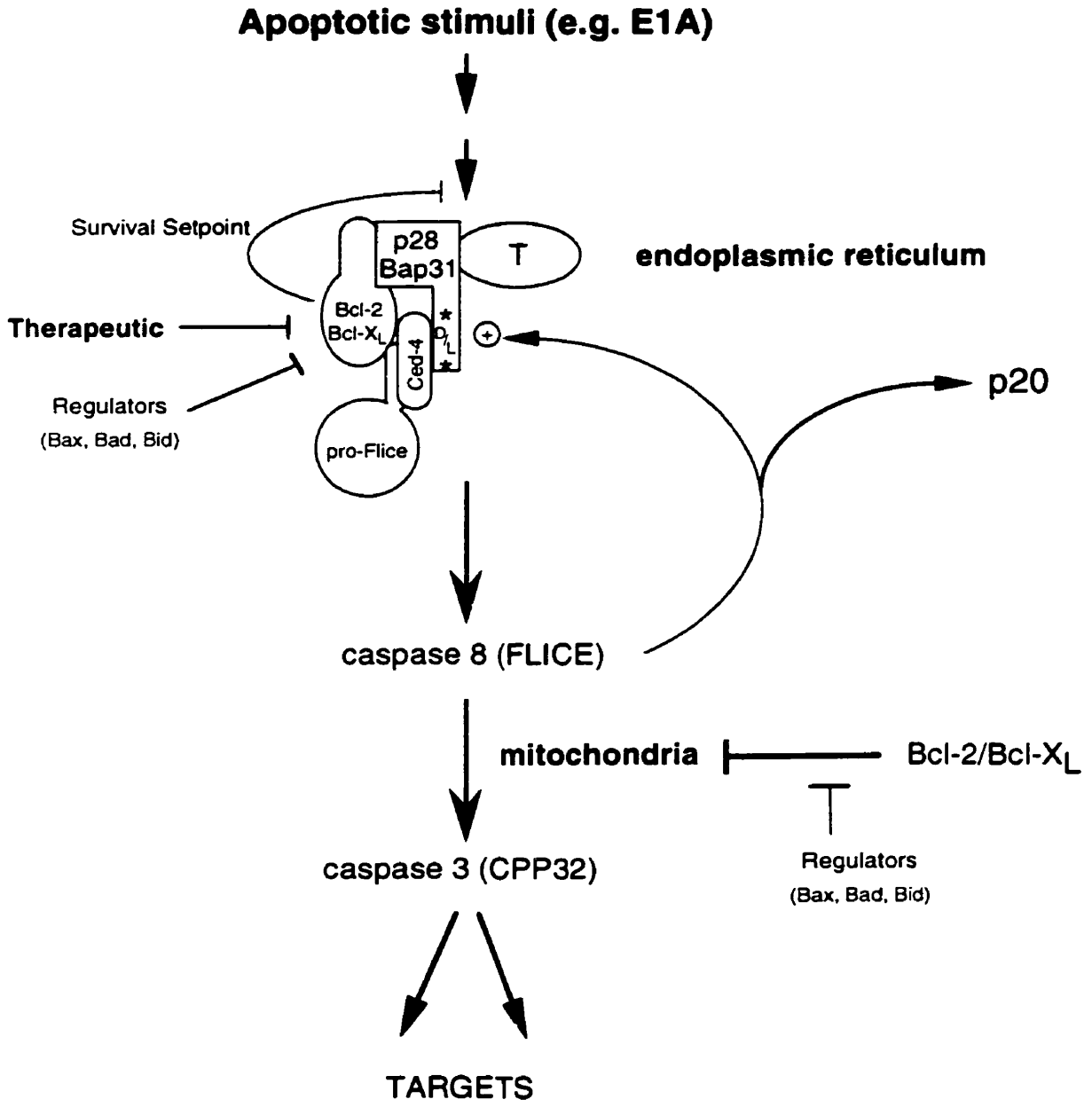
and AIF, and thus the activation of effector protease caspase-3 (Kluck et al., 1997a,b; Susin et al., 1997; Yang et al., 1997), it is conceivable that the presence of Bcl-2 in both organelles is to allow a complete inhibition of the caspase cascade by suppression the activation of both initiator (e.g. caspase-8) and effector (e.g. caspase-3) caspases. Furthermore, the activities of Bcl-2/Bcl-X_L proteins can be regulated Bax, Bad and Bid (Fig. 1).

In conclusion, p28Bap31 is identified and illustrated as a new player in the apoptotic pathway. The significance of this study is three fold. First, it is the first Bcl-2/Bcl-X_L interacting protein which also associates with the caspases and mammalian Ced-4 like molecule *in vivo*. Second, p28Bap31 is the first, and thus far only, cellular substrate identified for caspase-8 and its cleavage product, p20, shows a direct effect on the cell death pathway. Finally, its association with Bcl-2/Bcl-X_L, procaspase-8 and Ced-4/Ced-4-like molecule suggests a novel apoptotic regulatory complex presence in the ER which may be analogous to the Fas/TNFR1 death-inducing signaling complexes.

Nevertheless, there are questions which remain to be addressed. First, it is essential to assess the physiological role of p28Bap31 in cells. Ablation of p28Bap31 in animal models will provide invaluable information regarding the function of the protein. If the cleavage of p28Bap31 is crucial with respect to the activation of initiator caspases, removal of the protein would impede the ability of cells to engage cell death. It is, of course, also possible that p28Bap31 has other biological functions as well. It is also important to identify other molecules which participate in the p28Bap31 complex. These molecules will be of great interest for elucidating the precise mechanism of how this complex is involved in the regulation of apoptosis.

Figure 1. Proposed model.

In the absence of Bcl-2/Bcl-X_L, the p28Bap31/Ced-4/procaspase-8 complex is disrupted by apoptotic signals which cause the release and the activation of procaspase-8. Activated caspase-8 in turn triggers the caspase cascade by activating downstream caspases. p28Bap31 is cleaved to p20 which may function to amplify the caspase cascade or simply activate a parallel death pathway. This event at the ER may couple with the signals from mitochondria, which lead to the activation of effector caspase-3, to ensue an irreversible destruction of the cellular network. The presence of Bcl-2/Bcl-X_L in both organelles may inhibit the activation of the caspases and thereby suppress apoptosis. The activities of Bcl-2/Bcl-X_L proteins may be regulated by other apoptotic regulators such as Bax, Bad and Bid.


Apoptotic stimuli (e.g. E1A)
Survival Setpoint
p28
Bap31
T
endoplasmic reticulum
Therapeutic
Bcl-2
Bcl-X$_L$
Ced-4
D$_L$
Regulators
(Bax, Bad, Bid)
pro-Flice
p20
caspase 8 (FLICE)
mitochondria
Bcl-2/Bcl-X$_L$
Regulators
(Bax, Bad, Bid)
caspase 3 (CPP32)
TARGETS

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Contributions to original knowledge

1. p28Bap31 is a Bcl-2-associating protein which also interacts with Bcl-X_L, both *in vitro and in vivo*, but not with Bax.

2. *In vivo*, procaspase-8 is recruited to p28Bap31,through an adaptor molecule, which allows the cooperatively binding of Bcl-X_L to the carboxy half of p28Bap31.

3. *C. elegans* adaptor molecule Ced-4 can interact with p28Bap31. A carboxy terminal deletion mutant of Ced-4 distrupts the binding of Bcl-X_L to p28Bap31 *in vivo* indicating that a mammalian Ced-4-like molecule may be part of the p28Bap31/Bcl-X_L/procaspase-8 complex.

4. *In vitro*, p28Bap31 is cleaved by purified caspase-8 and -1, but not caspase-3. To date, p28Bap31 is the first and only cellular target of the initiator caspase-8.

5. *In vivo*, in the absence of Bcl-2 protein, p28Bap31 is cleaved at two caspase cleavage sites during apoptosis. The presence of Bcl-2 can prevent apoptosis and block the proteolytic cleavage of p28Bap31.

6. Ectopic expression of the large cleavage product of p28Bap31, p20Bap31 (p20), can induce apoptotic cell death which can be rescued by co-expression of Bcl-2.

7 p28Bap31 is an endoplasmic reticulum protein which is inserted into the membrane in N_{lumen}-C_{cyto} orientation leaving the protease sensitive carboxy half of the protein facing the cytoplasm.

8. p28Bap31 exists as a homodimer or oligomer *in vivo*.