Regulation of Apoptosis by the BAP31 Complex at the Endoplasmic Reticulum

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To obtain knowledge, add things everyday.

To obtain wisdom, remove things everyday.

Lao-tse, *Tao te Ching*

Abstract

Apoptosis is a genetically programmed, biochemically executed form of cell suicide essential for development and homeostasis in all multicellular organisms. The caspase family of cysteine proteases orchestrate the apoptosis program and are regulated by members of the Bcl-2 family. Extensive studies in mammals have revealed that Bcl-2 proteins control caspase activation by governing a key mitochondrial checkpoint, involving the permeabilization of the outer mitochondrial membrane and the release of proapoptotic factors from the intermembrane space. Bcl-2 proteins are located at other intracellular membranes, including the endoplasmic reticulum, and emerging evidence suggests that this organelle also plays a key role in the initiation of apoptosis. Here, I identify BAP31, a Bcl-2 associated protein of the ER membrane, as a central regulator of diverse apoptosis pathways. In response to signaling by the model oncogene, E1A, BAP31 and its cellular homolog and heterodimerizing partner, BAP29, were found to recruit and activate a novel procaspase-8L isoform at the surface of the ER by a BCL-2-inhibitable mechanism. Cells deficient in Bap31 and Bap29 resisted E1A-induced procaspase-8L activation, downstream effector caspase activation, and apoptosis, suggesting that the BAP/procaspase-8L complex plays a critical role in activating the apoptosis program following oncogenic stress. In addition to being a regulator of procaspase-8L, BAP31 was also found to be an important caspase-8 substrate. Caspase-8-induced mitochondrial dysfunction and apoptosis and were strongly inhibited by the expression of a caspase-resistant BAP31 mutant that remained structurally intact during apoptosis. Moreover, the p20 caspase cleavage fragment of BAP31 was found to induce Ca^{2+} transmission between the ER and mitochondria, which stimulated the onset of

mitochondrial fission and ultimately sensitized this organelle to caspase-8-driven cytochrome c release. Thus, BAP31 appears to be a cell death integrator that couples oncogenic signals to procaspase-8L activation, and triggers Ca^{2+}-dependent proapoptotic cross-talk between the ER and mitochondria.

Résumé

L'apoptose est un programme de suicide cellulaire génétiquement programmé qui est essentiel pour le développement et l'homéostasie des organismes multicellulaires. Une famille de protéases, nommées caspases, jouent un rôle central dans l'exécution de ce processus. L'activation des caspases est quant à elle régulée par la famille des homologues de BCL-2. Ds études extensives chez les mammifères ont révélé que les homologues de BCL-2 contrôlent l'activation des caspases en régulant l'intégrité de la membrane externe de la mitochondrie. En effet, au cour du processus apoptotique, plusieurs facteurs proapoptotiques sont relâchés de l'espace intermembranaire de la mitochondrie, résultant en l'activation des caspases et l'exécution de la cellule. Les homologues de BCL-2 sont aussi présent dans d'autres organelles, comme le réticulum endoplasmique, et de récentes données suggèrent que cette organelle joue un rôle important dans l'initiation de l'apoptose. Dans ce travail, j'ai identifié BAP31, une protéine du réticulum endoplasmique associée à BCL-2, comme étant un régulateur central de diverses voies apoptotiques. En réponse à l'activation de l'oncogène E1A, BAP31 et BAP29 (une protéine hautement homologue avec laquelle BAP31 interagit), recrutent un nouvel isoforme de la caspase-8 (procaspase-8L) à la surface du réticulum endoplasmique et l'activent par un mécanisme pouvant être inhibé par BCL-2. Ceci joue un rôle critique dans la voie de signalisation apoptotique de E1A car les cellules déficientes en BAP31 et BAP29 ne peuvent ni activer la procaspase-8L en présence de E1A, ni activer les caspases effectrices et la phase d'exécution. En plus de réguler la procaspase-8L, BAP31 est un substrat important de la caspase-8. L'expression d'un mutant de BAP31 ne pouvant être clivé par les caspases inhibe de façon importante les

événements liés à l'activation de la caspase-8 tels la perméabilisation de la membrane externe de la mitochondrie et la morphologie apoptotique. De plus, p20, le fragment apoptotique de BAP31, induit des signaux de Ca^{2+} qui permettent la communication entre le réticulum endoplasmique et les mitochondries. Ces signaux stimulent la fission mitochondriale et sensibilisent cette organelle à la sortie du cytochome c induite par la caspase-8. Ainsi, BAP31 semble être un intégrateur apoptotiques qui couple les signaux oncogéniques à l'activation de la procaspase-8 et régule d'important signaux liés au calcium entre le réticulum endoplasmique et les mitochondries.

Thanks to...

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Abbreviations

aa	amino acid
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
ATP	adenosine 5'-triphosphate
BAP	B cell receptor associated protein
Bcl	B cell lymphoma
BFA	brefeldin A
Caspase	cysteine aspartly protease
CsA	cyclosporine A
cyt.c	cytochrome c
CARD	caspase activation and recruitment domain
DD	death domain
DED	death effector domain
DECC	death effector coiled coil
DISC	death inducing signaling complex
DN	dominant-negative
ER	endoplasmic reticulum
FADD	fas associated death domain
GST	glutathione-S-transferase
HSP	heat shockl protein
IMM	inner mitochondrial membrane
IMS	intermembrane space
IP_3	inositol-1,4,5-trisphophate
IP_3R	inositol-1,4,5-trisphophate receptor
MMP	mitochondrial membrane permeabilization
MEF	mouse embryonic fibroblast
OMM	outer mitochondrial membrane
PTP	permeability transition pore
RyR	ryanodine receptor
SR	sacroplasmic reticulum
SERCA	sacroplasmic endoplasmic calcium ATPase
SMAC	second mitochondrial-derived activator of caspase
SREBP	sterol regulatory element binding protein
TG	thapsigargin
TM	transmembrane
TN	tunicamycin
TNF	tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
UVB	Utaviolet B
UPR	unfolded protein response

Contributions of Authors

This thesis includes the text and figures from five published research articles. I was first author on two of these manuscripts and contributed substantially, experimentally and intellectually, to the other three, which have been included for completeness. Parts of Chapters 1 and 6 (General Introduction and General Discussion) have been taken from two published review articles and a book chapter that I authored. The published material has been formatted to fit the style of the thesis and for convenience I have placed the references from all the chapters into one reference section at the end.

Chapter 2:

Breckenridge, D.G., Nguyen, M., Kuppig, S., Reth, M., and Shore, G.C. (2002). The procaspase-8 isoform, procaspase-8L, recruited to the BAP31 complex at the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *99*, 4331-4336.

While almost all the work in this paper was done by myself, much credit must be given to Mai Nguyen, who created the crBAP31-Flag stable cell lines and who originally observed the 62 kDa procaspase-8L protein associated with crBAP31-Flag. Mai and I worked closely together when I started in the lab and so the experiments shown in Figure 2.1 are representatives of many experiments conducted by the two of us while optimizing the IP conditions. It should also be noted that it was Stefan Kuppig who designed and carried out the elegant strategy to create the *Bap31*-null, *Bap29*-null, and *Bap29,Bap31*-null ES cells.

Chapter 3:

Nguyen, M., Breckenridge, D.G., Ducret, A., and Shore, G.C. (2000). Caspase-resistant BAP31 inhibits Fas-mediated apoptotic membrane fragmentation and release of cytochrome c from mitochondria. Mol. Cell. Biol. *20*, 6731-6740.

Most of the experiments done for this paper were conducted by Mai Nguyen. I helped repeat and confirm many of the experiments and contributed intellectually to the interpretation of the results. In addition, I conducted the experiment in Figure 3-4.

Ducret, A., Nguyen, M., Breckenridge, D.G., and Shore, G.C. (2003). The resident endoplasmic reticulum protein, BAP31, associates with gamma-actin and myosin B heavy chain: analysis by capillary liquid chromatography microelectrospray tandem mass spectrometry. Eur. J. Biochem. *270*, 342-249.

This paper was a joint effort between myself, Mai Nguyen and Axel Ducret. Mai carried out the immunoprecipitation experiments in Figure 3.9. Axel Ducret conducted the proteomic characterization of the immuno-isolated BAP31 complex (Figure 3.10, 3.11). I confirmed the specificity of the association between BAP31 and the actomysoin network in Figure 3.12.

Chapter 4:

Breckenridge, D.G., Marcellus, R.C., and Shore, G.C. Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals. J. Cell Biol. in press.

All of the experiments in this paper were conducted by myself. The AdMFpk$_3$Flice and Adp20 constructs were created and kindly provided by Marina Stojanovic and Richard Marcellus, respectively.

Chapter 5:

Wang, B.W., Nguyen, M., Breckenridge, D.G., Stojanovic, M., Clemons, P.A., and Shore, G.C. BAP31 requires the associated A4 protein to inhibit Fas-induced release of cytochrome c from mitochondria in intact cells. J. Biol. Chem. In Press.

Only part of the material covered in this paper was included in my thesis, which I combined with some of my own unpublished data. I conducted the experiments in Figure 5-1D, and Figure 5-3. Mai Nguyen conducted the experiments in Figure 5-2 and 5-4. The text is partially derived from this publication and partially rewritten by myself.

Chapters 1 and 6:

Breckenridge, D.G., Germain, M., Nguyen, M., Mathai, J., and Shore, G.C. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene, in Press.

Breckenridge, D.G., and Shore, G.C. (2002). Regulation of apoptosis by the endoplasmic reticulum. In Genetics of Apoptosis, S. Grimm, ed. (Oxford: BIOS Scientific Publishers Ltd.), pp. 93-112.

Breckenridge, D.G., and Shore, G.C. (2000). Regulation of apoptosis by E1A and Myc oncoproteins. Crit. Rev. Eukaryot. Gene Expr. *10*, 273-280.

Chapter 1

General Introduction

1.1 Apoptosis

To develop properly, maintain homeostasis, and combat environmental stresses, metazoans require a means to remove unwanted or damaged cells that are hazardous to the organism as a whole. This is achieved by an intrinsic cell suicide program called apoptosis, or programmed cell death, which rapidly destroys unwanted cells in an organized manner and leaves the potentially toxic cellular contents to be engulfed and degraded by neighboring cells. During development, apoptosis is used as a tool to sculpt tissues and organs (Meier et al., 2000). Animals overproduce cells during organ development and tissue architecture is established by selectively removing certain cells; for example, in the interdigit web regions of hands and toes. Soluble proapoptotic cytokines such as Tumor Necrosis Factor-α (TNFα) and death ligands, such as Fas, present on the surface of cytotoxic T-cells, survey the peripheral immune system and trigger apoptosis in unstimulated lymphocytes and virus infected cells (Krammer, 2000). Excessive stress to individual cells also triggers the innate cell suicide program. For example, extensive DNA damage or uncontrolled cell proliferation are sensed by the tumor suppressor p53, which then engages the core apoptotic machinery (Vogelstein et al., 2000). Cell detachment from the extracellular matrix, low growth factor conditions, and compromisation of protein folding processes are other examples of stresses that induce apoptosis.

Given that apoptosis plays a critical role in development, homeostasis, and defense processes, it is not surprising that mutations in genes that initiate and execute apoptosis are implicated in many diseases (Kam and Ferch, 2000). Mutations that inhibit apoptosis can offset the delicate balance between cell growth and cell death, causing

conditions such as cancer. In contrast, inappropriate activation of apoptosis results in the destruction of vital cells and can cause neurodegenerative disorders (Yuan and Yankner, 2000). Apoptosis is also implicated in the pathology of many diseases including stroke, AIDS, and Alzheimer's disease (Kam and Ferch, 2000; Wellington and Hayden, 2000). The ability to tip the balance in favor or against the initiation of apoptosis could, therefore, provide a therapeutic entrée to cure many of these diseases (Nicholson, 2000). A prerequisite for such therapeutic strategies, however, is a strong understanding of the basic cellular pathways that control apoptosis.

1.2 Morphological Characteristics of Apoptosis

Apoptosis is typically characterized by a number of morphological changes that represent the various cell disassembly processes and culminate in the coordinated packaging of cellular contents into apoptotic bodies – fragmented, but membrane enclosed cell particles. Apoptotic bodies are rapidly eliminated by neighboring epithelial cells or macrophages, which ensures that an inflammatory response is avoided (Savill and Fadok, 2000). Early morphological features of apoptosis include cell detachment and rounding, plasma membrane blebbing, and loss of phosphatidyl serine asymmetry at the plasma membrane (reviewed in Mills et al., 1999). Cell rounding and detachment are caused by rearrangement of focal adhesion complexes and myosin-dependent reorganization of the actin fibers into a peripheral ring around the plasma membrane. Plasma membrane blebbing then occurs at sites where membrane adhesion to the contracted actin network is lost. Phosphatidyl serine flippage to the outer leaflet of the plasma membrane, on the other hand, is a specific "eat me" signal that activates the cell

corpse engulfment process (Savill and Fadok, 2000). Other early features of apoptosis include the collapse of the nuclear membrane and chromosome condensation. Several endonucleases then induce intranucleosomal DNA fragmentation, which ensures the destruction of viral or mutated genomic DNA (Hengartner, 2000). Eventually, actin fibers and microtubules are degraded, cytosolic volume is lost, and the cell shrinks and fragments into apoptotic bodies.

1.3 Programmed Cell Death in *Caenorhabditis elegans*: an Introduction to Caspases and Bcl-2 Proteins.

Elegant genetic studies in the nematode, *C. elegans*, created a simple molecular framework for understanding the regulation of programmed cell death. Cell death is regulated by four genes: *egl-1*, *ced-3*, *ced-9*, and *ced-4* (Metzstein et al., 1998). Loss of function mutations in *egl-1*, *ced-3,* and *ced-4*, or gain of function mutations of *ced-9*, abrogate cell death. Therefore, *egl-1*, *ced-3*, and *ced-4* promote cell death, whereas *ced-9* is an inhibitor of cell death. Genetic and molecular characterization of these genes and their respective proteins has unveiled a linear pathway leading to cell death; EGL-1 antagonizes CED-9, CED-9 antagonizes CED-4, and CED-4 activates CED-3 (Fig. 1.1). EGL-1 is transcriptionally unregulated in response to developmental cues (Conradt and Horvitz, 1999) and binds CED-9, which is precomplexed with CED-4 at the surface of mitochondria (Chen et al., 2000). CED-4 is released from CED-9, and now oligomerizes and activates CED-3. CED-3, in turn, induces all of the downstream the morphological features of apoptosis (Yuan and Horvitz, 1990). Identification of mammalian homologs of these proteins revealed that CED-9 is a member of the multidomain antiapoptotic Bcl-2

family and EGL-1 is a member of the BH3-only subset of proapoptotic Bcl-2 proteins (Metzstein et al., 1998) (see section 1.7.2). CED-3, on the other hand, is a caspase; aspartate-specific cysteine proteases that induce the morphological features of apoptosis by cleaving select substrate molecules (see section 1.4). CED-4 is homologous to Apaf-1, a mammalian caspase activating protein (section 1.6.2). Therefore, the apoptosis program in *C. elegans* provides a simple paradigm for understanding how Bcl-2 proteins govern the activation of caspases during apoptosis.

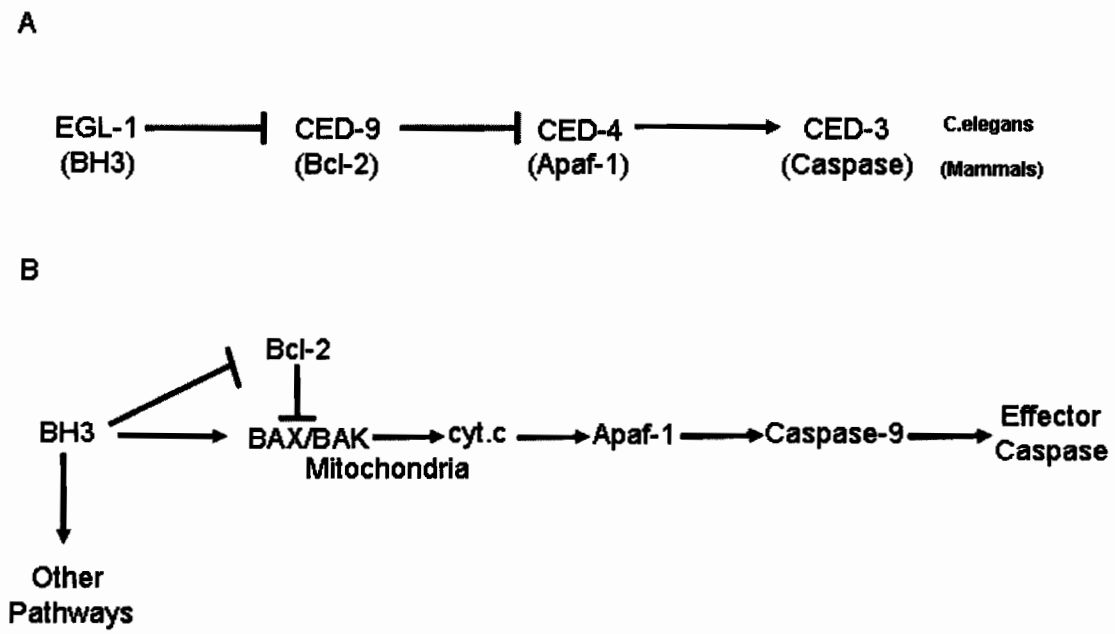

Figure 1.1. Apoptosis pathways in *C.elegans* and mammals. In *C. elegans*, EGL-1 induces apoptosis by binding and inhibiting CED-9, causing the release of CED-4 from CED-9, and allowing activation of CED-3. Mammalian homologs have been identified for each of these proteins (in parathesis). The central death pathway is conserved in mammals but involves an additional regulator step involving the release of cytochrome c (cyt.c) from mitochondria (described in section 1.6.2 and 1.7).

1.4 The Caspase Family

Caspases hydrolyze proteins on the C-terminal side of Asp residues lying within a tetrapeptide recognition sequence - specificity is normally conferred by the three amino acids on the N-terminal side of the Asp residue (Nicholson, 1999). All caspases are

synthesized as inactive zymogens (procaspases) containing an N-terminal regulatory prodomain and a large (approximately 20 kDa) and a small (approximately 10 kDa) enzymatic subunit (Fig. 1.2). Caspase recognition sequences exist between each of these domains, permitting proteolytic activation to occur either by self-processing or through cleavage by another caspase. This separates the three domains, the large and small subunits heterodimerize and assemble into heterotetrameric haloenzyme (Hengartner, 2000). In most cases, the activation of caspases is thought to represent the point of no return during apoptosis. Consistent with this, inhibition of caspases with small peptide inhibitors, such as the pancaspase inhibitor, zVAD-fmk, or targeted disruption of certain caspase genes can prevent apoptosis (Green and Kroemer, 1998; Zheng et al., 1999).

The 14 identified caspases in mammals can be divided into three classes based on their sequence similarity, function, and substrate specificity (Earnshaw et al., 1999). Initiator caspases couple diverse death signals into a cascade of caspase activation, and include procaspase-2, procaspase-8, procaspase-9, and procaspase-12. These procaspases have long prodomains containing homotypic interaction motifs, such as the Death Effector Domain (DED) or the Caspase Activation and Recruitment Domain (CARD), which mediate recruitment into activation complexes and autolytic processing. Following activation, initiator caspases cleave and activate downstream effector caspases (Fig. 1.2). Caspase-8 and 9 have a preference for (I/V/L)EXD sequences (Thornberry et al., 2000), although exceptions exist (Ng et al., 1997; Stegh et al., 2000).

Effector caspases have short prodomains lacking regulatory sequences and are activated upon cleavage by upstream initiator caspases. Effector caspases include caspase-3, -6, and -7. These caspases invoke the execution stage of apoptosis by cleaving a diverse array of cellular proteins at the consensus sequence DEXD (Thornberry et al.,

2000). Cleavage of each substrate somehow partakes in a cell disassembly process, the net effect of these processes gives rise to the coordinated destruction of the cell (Earnshaw et al., 1999).

Inflammatory caspases, as their name suggests, are not involved apoptosis, but rather mediate inflammatory responses. Inflammatory caspases include caspase-1, -4, -5

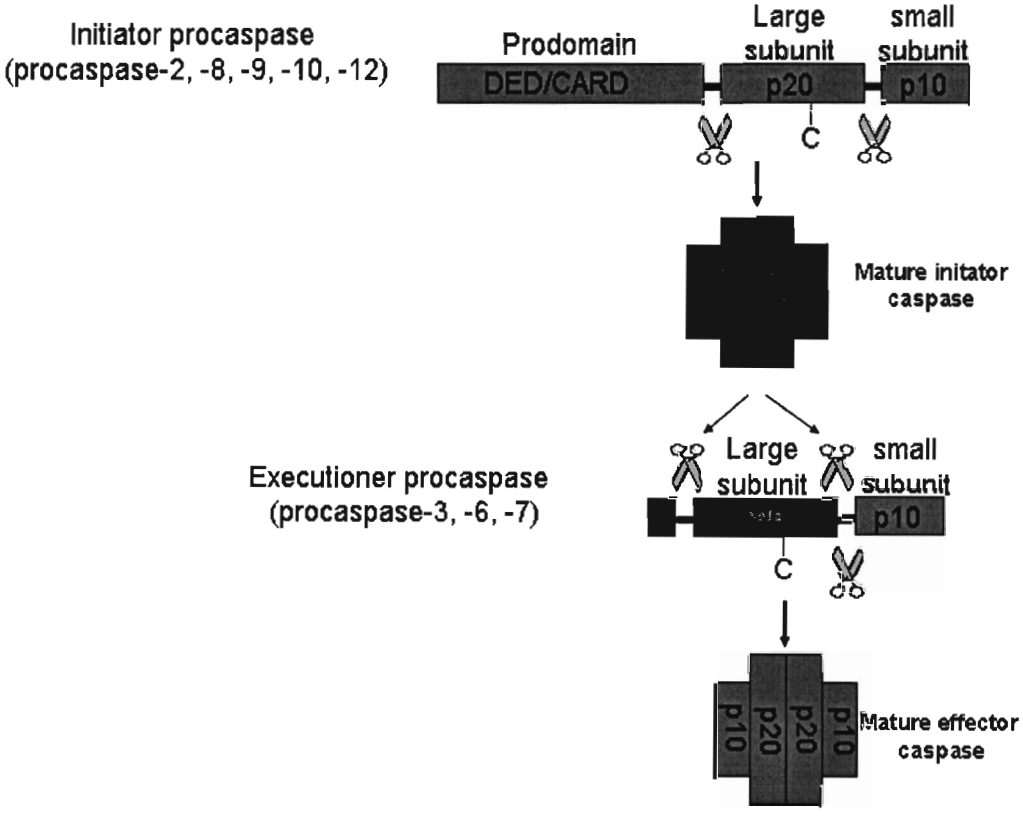

Figure 1.2. The caspase family. Initiator procaspases have long prodomains containing sequences, such as the Death Effector Domain (DED) or Caspase Activation and Recruitment Domain (CARD), which mediate procaspase recruitment to activation complexes. Initiator procaspases normally undergo autocatalytic activation and subsequently cleave and activate downstream effector caspases. The active form of all caspases is a heterotetramer of the p20 and p10 subunits.

(murine caspase-11), and -13, although a functional role for caspase-4 and -13 in inflammation has not been established (Earnshaw et al., 1999; Martinon et al., 2002).

Finally, caspase-14 appears to mediate epidermal keratinocyte differentiation (Kuechle et al., 2001; Lippens et al., 2000).

1.5 Caspase Substrates

Caspase substrates can be generally broken into two categories depending on which type of caspases they are cleaved by. The vast majority are executioner caspase substrates (executioner substrates) that trigger the different cell disassembly processes. Since over 100 executioner substrates have been identified (Hengartner, 2000), I will restrict the following section to the description of a few well characterized examples.

Caspase cleavage of a protein can result in four outcomes: 1) direct inactivation of a protein; 2) inactivation of a protein by generating a dominant negative cleavage product that antagonizes the full-length protein; 3) direct activation of a protein by removal of a regulatory domain; or 4) indirect activation of a protein by inactivating a regulatory subunit (Hengartner, 2000). Caspase-6 cleavage of nuclear lamins is an example of direct inactivation of protein by caspases (Ruchaud et al., 2002). Cleavage of lamins during apoptosis structurally dismantles the lamin network and causes chromatin condensation. In contrast, caspase-3 cleavage of BCL-2 and BCL-xL generates dominant interfering cleavage products that prevent the antiapoptotic activity of full-length Bcl-2 and Bcl-xL (Cheng et al., 1997; Clem et al., 1998). On the other hand, caspase-3 cleavage of ROCK-1, a serine/threonine kinase, removes its C-terminal inhibitory domain and generates a constitutively active kinase (Sebbagh et al., 2001). Caspase-cleaved ROCK-1 stimulates Myosin-II phosphorylation and activity, causing actin contraction and apoptotic membrane blebbing. Finally, caspase-3-mediated cleavage of ICAD provides an example

of indirect activation of an enzyme by inactivation of a regulatory subunit (Nagata, 2000). Caspase-Activated DNase (CAD) is normally kept inactive by its inhibitor, ICAD. Cleavage of ICAD releases CAD, which can now partake in apoptotic DNA degradation.

Initiator substrates are cleaved by initiator caspases. Aside from executioner procaspases, very few initiator substrates have been identified to date. The substrate specificity of caspase-8 has been studied the most extensively. Following its activation by the TNF family of death receptors at the plasma membrane, caspase-8 directly cleaves procaspase-3 (Blanchard et al., 2000). However, in many cell types, caspase-8 signals must be amplified through a mitochondrial loop (Scaffidi et al., 1998). This is achieved by caspase-8 cleavage of BID, a BH3-only protein of the Bcl-2 family. The resulting truncated BID (tBID) fragment translocates to mitochondria and induces the release of intermembrane space (IMS) proapoptotic factors, which further stimulate caspase activation in the cytosol (see section 1.5) (Li et al., 1998; Luo et al., 1998). BAP31 is another key caspase-8 target (Ng et al., 1997) that activates Ca^{2+}-dependent crosstalk between the ER and mitochondria. These ER Ca^{2+} signals seem to cooperate with tBID to induce the release of proapoptotic factors from mitochondria (Breckenridge et al., 2003). Caspase-8 cleavage of BAP31 is the focus of much of this thesis (see Chapters 3-5). Finally, caspase-8 cleaves plectin, a cytolinker that crosslinks all three cytoskeletal filament systems (Stegh et al., 2000). This is thought to initiate the hierarchal disruption of the cytoskeleton. Thus, initiator caspases cleave an exclusive group of proteins, which in turn facilitate executioner caspase activation and the progression of apoptosis.

Other intracellular proteases can also regulate apoptosis. For example, granzyme B, a serine protease exocytosed by cytotoxic T lymphocytes and taken up by target cells, can activate apoptosis by directly cleaving procaspase-3 and BID (Darmon et al., 1995;

Heibein et al., 2000). Calpains have been implicated in activating certain caspases and may cooperate with caspases during the execution phase of apoptosis (Wang, 2000; Waterhouse et al., 1998; Wood and Newcomb, 1999). Cathepsins have also been implicated in apoptosis (Turk et al., 2000), although the precise role of these lysosomal proteases during cell death is unclear.

1.6 Initiator procaspase activation

1.6.1 The Death Inducing Signaling Complex

The best studied, and prototypical, caspase activation complex is the Death Inducing Signaling Complex (DISC) formed by the TNF family of death receptors at the plasma membrane. This family includes TNF receptor 1 (TNF-R1), Fas/CD95, and the TNF Related Apoptosis Inducing Ligand (TRAIL) receptors-1 and -2 (TRAIL-R1 and TRAIL-R2) (Baud and Karin, 2001). Following binding to their cognate cytokine ligands (or to agonistic antibody in experimental systems) these receptors trimerize and recruit and activate procaspase-8 (Fig. 1.3). In each case, this is mediated by a homotypic Death Domain (DD) interaction between the intracellular portion of the receptor and the adapter molecule FADD. In the case of TNF-R1, FADD is recruited indirectly via a second DD-containing adapter called TRADD. FADD also contains a DED that recruits procaspase-8 to the receptor DISC (Medema et al., 1997; Muzio et al., 1996). The induced proximity of many procaspase-8 molecules at the DISC triggers autocatalytic activation and the mature caspases are released to the cytosol (Muzio et al., 1998). Procaspase-10 is also recruited to the DISCs, however, it is unclear whether it plays an essential role in death receptor pathway (Kischkel et al., 2001; Sprick et al., 2002; Xiao et al., 2002).

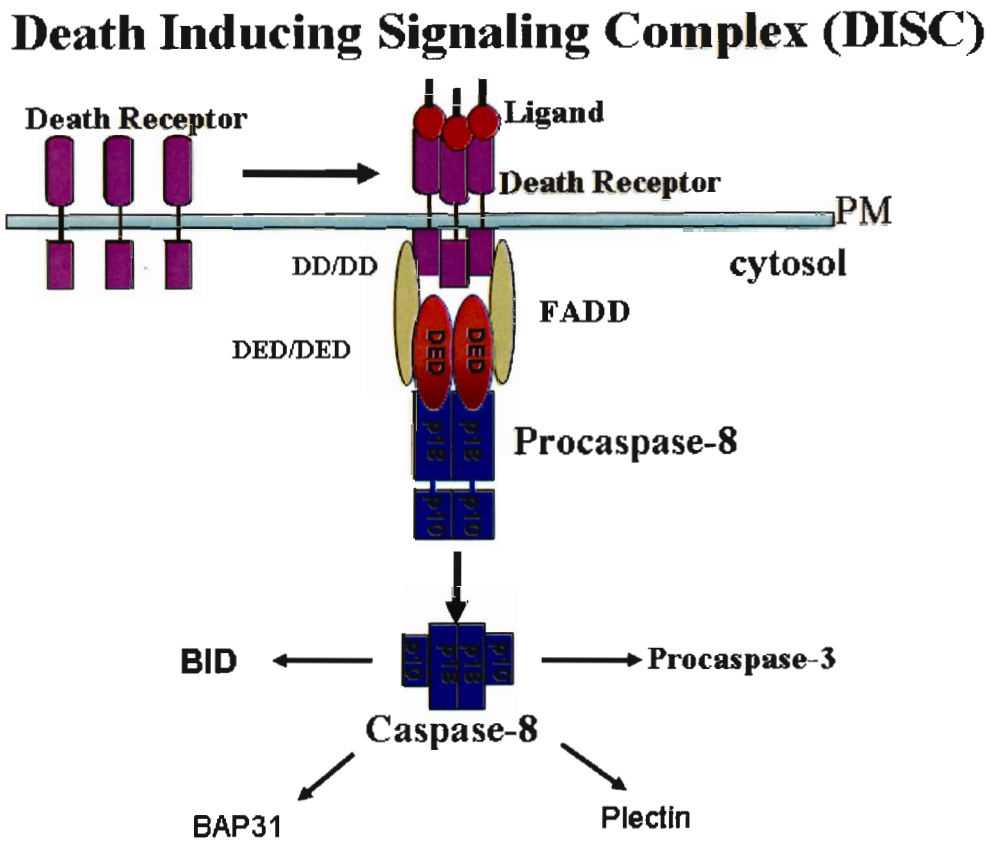

Figure 1.3. The Death Inducing Signaling Complex. Death receptors are activated upon ligand binding, which induces receptor trimerization and recruitment of procaspase-8 via FADD. The induced proximity of many procaspase-8 molecules induces autolytic processing and release of the mature caspase. Caspase-8 then cleaves select substrates, including procaspase-3, BID, BAP31 and plectin.

1.6.2 The Apoptosome

A common event in most apoptosis pathways is the limited permeabilization of the outer mitochondrial membrane (OMM) and subsequent release of cytochrome c (cyt.c) to the cytosol. cyt.c is normally located in the intermembrane space (IMS) of the mitochondrion, where it shuttles electrons between complex III and IV of the electron transport chain (De Santis and Melandri, 1984; Kadenbach, 1986). However, in the cytosol, cyt.c binds to the CED-4 homolog, Apaf-1, and triggers the assembly of a

cyt.c/Apaf-1/procaspase-9 complex called the apoptosome (Budihardjo et al., 1999) (Fig 1.4). A number of biochemical studies suggest that binding of cyt.c to the WD40 repeats of Apaf-1 induces dATP-dependent oligomerization of Apaf-1 and exposure of its CARD, which recruits procaspase-9 to the complex via a homotypic CARD interaction. The three dimensional structure of the apoptosome resembles a seven-spoked wheel; seven Apaf-1 molecules make up the spokes, and seven cyt.c and procaspase-9 molecules form the hub region (Acehan et al., 2002). Procaspase-9 does not appear to undergo proteolytic activation; rather, the proenzyme remains associated with the apoptosome and undergoes a conformational change that allows it to cleave and activate procaspase-3 and-6.

1.6.3 Procaspase-2 Activation Complex

Recently it was reported that procaspase-2 is recruited into an approximately 700 kDa cytosolic complex that mediates its activation (Read et al., 2002). Although similar in size, the caspase-2 activation complex does not contain components of the apoptosome. However, like procaspase-9, procaspase-2 may not require proteolytic cleavage to stimulate enzyme activity (Read et al., 2002). The molecular constituents of caspase-2 complex are unknown at present.

1.6.4 The Inflammasome

Although not involved in apoptosis, the recently identified inflammasome complex provides another example of a caspase activation complex (Martinon et al., 2002). The inflammasome is thought to receive foreign alarm signals and respond by generating mature caspase-1, which is responsible for the maturation of interleukin-

1β and interleukin-18 in phagocytes. Procaspase-1, procaspase-5, ASC and NALP1 make up the inflammasome (Martinon et al., 2002). NALP-1 receives signals from the Toll-like receptors and, in turn, recruits ASC through a homotypic PYRIN domain interaction. The CARD of ASC, in turn recruits procaspase-5 (caspase-11 in mouse) and procasapse-1 to the complex via homotypic CARD interactions. Although the details are not known at present, procaspase-5 presumably undergoes self-activation at the complex and then cleaves procaspase-1. Mature caspase-1 then clips prointerleukin-1β and -18, generating the mature cytokines, which are secreted from the cell (Tschopp et al., 2003).

1.6.5 The BAP31 Complex

BAP31 is not only a caspase-8 substrate but also a putative procaspase-8 activation complex at the ER. BAP31 and its cellular homolog and heterodimerizing partner, BAP29, are integral proteins of the endoplasmic reticulum (ER) membrane (Adachi et al., 1996; Mosser et al., 1994; Ng et al., 1997). Both proteins have a short N-terminal region in the lumen of the ER, three transmembrane (TM) domains, with a 37 amino acid lumenal loop existing between TM2 and TM3, followed by a cytosolic tail containing a coiled coil domain and terminating in a canonical KKXX ER retrieval sequence (Adachi et al., 1996; Mosser et al., 1994; Ng et al., 1997). BAP31 and BAP29 were originally discovered as immunoglobulin D associated proteins [and were thus called B cell receptor-associated proteins (Adachi et al., 1996; Kim et al., 1994)]. BAP31 was later shown to affect the transport of cellubrevin from the ER (Annaert et al., 1997) and the stability of the cystic fibrosis transmembrane conductance regulator (Lambert et al., 2001). More recently, BAP31 was reported to bind Class I MHC molecules at the ER

(Spiliotis et al., 2000) and enhance the rate of MHC I transport to the Golgi (Ladasky, 2002). These studies have implicated BAP31 as an ER cargo receptor or chaperone (Annaert et al., 1997). A role for BAP31 in apoptosis came from its discovery as a BCL-2/BCL-xL-associated protein (Ng et al., 1997). The coiled coil domain in BAP31 and BAP29 share low sequence identity with the DEDs of FADD and procaspase-8, and this region has been coined the Death Effector Coiled Coil (DECC) domain (Nguyen et al., 2000). In cotransfected 293T cells, BAP31interacts with procaspase-8, BCL-2 and BCL-xL, raising the possibility that BAP proteins can bridge Bcl-2 proteins to the regulation of procaspase-8 activation in certain cell death pathways (Ng et al., 1997; Ng and Shore, 1998). Regulation of procaspase-8 by BAP31 and BAP29 is discussed in more detail in section 1.8.4 and is the focus of Chapter 2.

1.7 Mitochondria and Apoptosis

Mitochondria integrate diverse cell death stimuli into a core apoptosis pathway (Green and Reed, 1998). This is achieved through mitochondrial release of several IMS proapoptotic factors, which either stimulate caspase activation or apoptotic DNA degradation (Fig. 1.4). Cyt.c was the first of these proteins to be discovered and, as described above, is an essential cofactor for the activation of procaspase-9. SMAC and HtrA2 are released from the mitochondrial IMS with cyt.c, and increase the activity of downstream effector caspases by binding and sequestering the Inhibitor of Apoptosis proteins (IAP) (Du et al., 2000; Hegde et al., 2002; Verhagen et al., 2000). IAPs normally bind the catalytic site of caspase 3, 7 and 9, ensuring that these caspases remain inactive in healthy cells (Budihardjo et al., 1999). Thus, mitochondrial release of SMAC

and HtrA2 guarantees that caspases become fully activated following the formation of the cyt.c/Apaf-1/caspase-9 apoptosome complex. Finally, mitochondria release AIF and endonuclease G, two proteins that cooperate to induce DNA degradation during apoptosis (Li et al., 2001; Parrish et al., 2001; Susin et al., 1999; Wang et al., 2002). Interestingly, endonuclease G is also involved in the replication of mitochondrial DNA (Cote and Ruiz-Carrillo, 1993), and AIF is a flavoprotein that may have a function in mitochondrial metabolism (Susin et al., 1999). Since most, if not all, IMS proteins are released from mitochondria during apoptosis (Van Loo et al., 2002), it remains possible that mitochondria harbor other, unidentified, activators of the different cell death disassembly processes.

The mechanism by which the OMM undergoes selective permeabilization (herein referred to as mitochondrial membrane permeabilization, MMP) is highly controversial and poorly understood (Zamzami and Kroemer, 2001). During apoptosis mitochondria appear to undergo a number of ultrastructural changes that are critical for MMP. For example, prior to MMP, the highly interconnected mitochondrial network fragments into small punctiform organelles. Youle and colleagues demonstrated that this is the result of a large-scale activation of the mitochondrial fission process (Frank et al., 2001). Inhibition of Drp1, a dynamin related protein that mediates mitochondrial fission, completely blocks apoptotic fragmentation of mitochondria and MMP (Frank et al., 2001). MMP also involves remodeling of cristae. Approximately 85% of cyt.c stores are normally trapped in the tight cristae folds of the inner mitochondrial membrane (IMM), where oxidative phosphorylation reactions occur. Prior to MMP, individual cristae fuse together, expanding the junction borders between cristae and the IMS (Scorrano et al., 2002). Apoptotic cristae remodeling might permit the mobilization of cyt.c out of these

junctions for transport across the OMM. Opening of the mitochondrial permeability transition pore (PTP) is also implicated in MMP, although the length of PTP opening and its role in MMP are controversial (Zamzami and Kroemer, 2001). The PTP is a high conductance non-selective ion channel that spans the IMM and OMM at points where the two membranes are in contact (Crompton, 1999). The voltage dependent anion channel

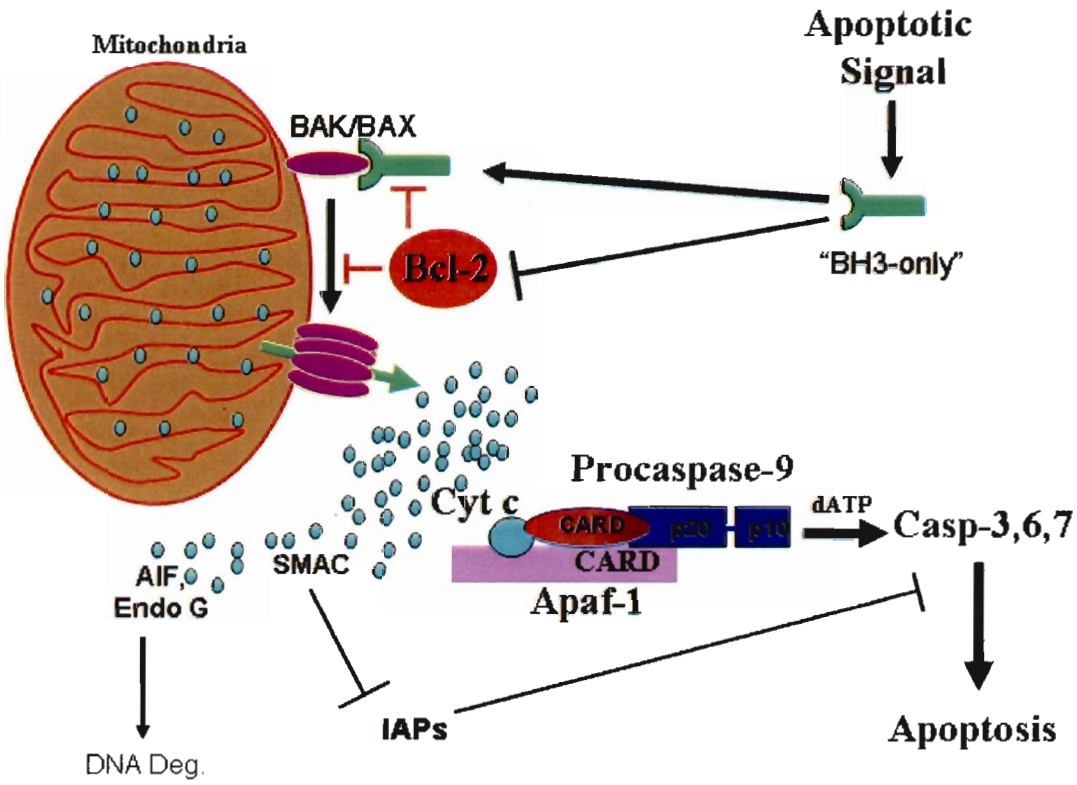

Figure 1.4. Regulation of MMP by Bcl-2 proteins. Following activation, BH3-only proteins translocate to mitochondria and induce the oligomerization of BAX and BAK in the OMM as well as other ultrastructural changes in the organelle. Bcl-2 sequester BH3-only proteins and inhibits the activation of BAX and BAK. BAX/BAK oligomers are thought to create pores in the OMM for the passage of IMS proteins. In the cytosol, cyt.c induces the formation of the apoptosome, and SMAC and HtrA2 sequester IAPs allowing full scale caspase activation. AIF and endonuclease G, on the other hand, translocate to the nucleus and induce DNA degradation.

(VDAC) located in the OMM, and the adenine nucleotide translocator (ANT), located in the IMM, are the core constituents of the PTP. Other components include matrix localized cyclosporine D (the target of the PTP suppressor, cyclosporine A [CsA]), hexokinase II, creatine kinase, and the benozodiazepine receptor. In some models, transient openings in the PTP are involved in cristae remodeling (Scorrano et al., 2002), whereas other models posit that components of PTP interact with Bcl-2 proteins to form cyt.c exit pores in the OMM (Zamzami and Kroemer, 2001). In either case, PTP opening is important for MMP because CsA has been shown to inhibit cyt.c release (reviewed in Zamzami and Kroemer, 2001).

In late stages of apoptosis the permeability of the inner mitochondrial membrane (IMM) is also lost, leading to dissipation of the mitochondrial electrochemical gradient ($\Delta\Psi_m$), matrix swelling, and rupture of the OMM (Green and Reed, 1998). These late steps, however, are thought to occur in a caspase-dependent fashion after the release of IMS proteins, and are thus not involved in MMP (Goldstein et al., 2000).

1.7.1 Regulation of MMP by Bcl-2 proteins.

MMP is directly regulated by the Bcl-2 family of proteins. Over 20 Bcl-2 proteins have been identified in mammals (reviewed in Adams and Cory, 2001; Antonsson and Martinou, 2000; Gross et al., 1999a; Martinou and Green, 2001). Overall, these proteins share low sequence homology, except within so-called Bcl-2 homology (BH) domains, which facilitate homodimerization and heterodimerization between the different subclasses of Bcl-2 proteins. Antiapoptotic multidomain Bcl-2 proteins contain four BH regions (BH1 to BH4) and include BCL-2 itself, BCL-xL, MCL-1, A1, BOO, BCL-W, and NR-13. Multidomain proapoptotic Bcl-2 proteins contain BH1, BH2, and BH3

domains and include BAX, BAK, and BOK. Finally, BH3-only members retain only the BH3 domain, are proapoptotic, and include BID, BAD, BIK, BIM, HRK, NOXA, PUMA, and NIP3. Multidomain Bcl-2 proteins, in addition to several BH3-only proteins, contain membrane insertion sequences that mediate targeting to the OMM, the endoplasmic reticulum, and nuclear membrane. Localization of Bcl-2 proteins to the different organelles is critical for their effect on apoptosis (Gross et al., 1999a; Nguyen et al., 1994).

An emerging theme in the literature is that BH3-only proteins couple diverse apoptotic signals to the activation of multidomain BAX and BAK (Korsmeyer et al., 2000). Bcl-2 antiapoptotic proteins prevent this activation by either sequestering BH3-only proteins in an inactive state, or by directly binding BAX and BAK and preventing conformational changes that lead to their activation. The three dimensional structures of BAX and BCL-xL (bound to a BH3 peptide) revealed that the BH1, BH2 and BH3 domains form a hydrophobic "acceptor" pocket that binds a free "donor" BH3 domain (Muchmore et al., 1996; Suzuki et al., 2000). It is, therefore, believed that BH3-only proteins are ligands for BAK and BAX receptors at intracellular organelles, and that Bcl-2 antiapoptotic proteins act as decoy receptors (Letai et al., 2002b). Consistent with this, mutation of the BH3 domain abrogates the ability of BH3-only proteins to bind BCL-2, BAX, and BAK, and induce apoptosis (Gross et al., 1999a). Recently, BH3-only proteins were further divided into two functional groups: BH3s that bind BCL-2 but not BAK or BAX, and BH3s that bind both BCL-2 and BAX and BAK (Letai et al., 2002b). One prediction is that the former group may counter BCL-2 repression by blocking the BH3 cleft of BCL-2, allowing the latter group of BH3s to bind and activate BAX and BAK.

BH3-only proteins are activated either by transcriptionally upregulation or by post-translational modification (reviewed in Puthalakath and Strasser, 2002). For example, BIK, PUMA and NOXA are transcriptional upregulated by p53 in response to oncogenic or genotoxic stress. As mentioned earlier, BID undergoes cleavage by caspase-8 during death receptor-mediated apoptosis. In contrast, BAD is phosphorylated in a growth factor dependent manner, which keeps it sequestered in the cytosol by 14-3-3 (Zha et al., 1996). Similarly, BIM is normally sequestered by the microtubule-associated dynamin motor complex, but is released to the mitochondria in response to certain death signals (Puthalakath et al., 1999). Overexpression of tBID, BAD, BIM, and NOXA does not induce apoptosis in *Bax,Bak*-double null cells (Cheng et al., 2001; Wei et al., 2001), which confirms that BH3-only proteins exert their effect through the multidomain proapoptotic Bcl-2 members. BAX forms pores in synthetic liposomes, suggesting that BAX and BAK homooligomers may form exit conduits in the OMM for the passage of IMS proteins during apoptosis (Korsmeyer et al., 2000; Zamzami and Kroemer, 2001) (Fig.1.4). Consistent with this idea, binding of tBID to BAX or BAK induces BAX/BAK homooligomerization in the OMM (Ruffolo et al., 2000; Wei et al., 2000) and causes cyt.c release (Wei et al., 2000). A recent report (Kuwana et al., 2002), demonstrated that tBID could stimulate BAX or BAK pores, in vesicles reconstituted from the OMM or purified lipids, capable of releasing 2000 kDa dextrans. As mentioned above, however, other ultrastructural changes in mitochondria, such as fission, cristae remodeling, and PTP opening, are required for MMP and at present it is unclear how Bcl-2 proteins regulate these processes.

Comparison of how Bcl-2 proteins function in *C. elegans* and in mammals suggests the functioning mechanisms of this family has changed significantly through

evolution. In the worm, CED-9 directly antagonizes a caspase activation molecule, CED-4, whereas in mammals Bcl-2 antiapoptotic proteins seem to inhibit MMP and, therefore, indirectly prevent caspase activation. One explanation for this conundrum is that in mammals Bcl-2 proteins also regulate caspase activation at other cellular loci, such as the ER.

1.8 Regulation of Apoptosis by Endoplasmic Reticulum Pathways

Emerging evidence suggests that the ER regulates apoptosis both by sensitizing mitochondria to a variety of extrinsic and intrinsic death stimuli and by initiating cell death signals of its own. Members of all three classes of the BCL-2 family localize to the ER membrane and have been shown to influence ER homeostasis, perhaps by influencing membrane permeability. Calcium release from the ER, for example, has been implicated as a key signaling event in many apoptotic models and, depending on the mode of Ca^{2+} release, may either directly activate death effectors or influence the sensitivity of mitochondria to apoptotic transitions. Furthermore, a growing number of ER proteins have been shown to influence apoptosis by either interacting with BCL-2 family members or altering ER Ca^{2+} responses, whereas several ER proteins are caspase substrates that may regulate the execution phase of apoptosis. Moreover, recent studies on how stress in the ER is coupled to apoptosis have demonstrated that the ER, like mitochondria, can directly initiate pathways to caspase activation and apoptosis.

1.8.1 ER stress-induced pathways

The endoplasmic reticulum is the first stop on the secretory pathway wherein chaperone-assisted polypeptide folding and modification ensures that proteins obtain their mature conformation. When the capacity of the ER to properly fold proteins is compromised or overwhelmed, a highly conserved unfolded protein response (UPR) signal transduction pathway is activated. The UPR halts general protein synthesis while upregulating ER resident chaperones and other regulatory components of the secretory pathway (Travers et al., 2000), giving the cell a chance to correct the environment within the ER (Patil and Walter, 2001). However, if the damage is too strong and homeostasis cannot be restored, the mammalian UPR ultimately initiates apoptosis. This switch, from metabolic arrest, which provides an opportunity for repair of the ER folding capacity, to cell death, which eliminates an overly damaged cell, is analogous to the p53 response to genotoxic stress. In contrast to the p53 switching mechanisms, however, the analogous processes relating to the ER remain poorly understood.

1.8.1.1 ER Stress: the Survival Response

In mammals, three ER transmembrane proteins, Ire1, ATF6, and PERK, respond to the accumulation of unfolded proteins in the lumen (see Kaufman, 1999; Patil & Walter, 2001; Sidrauski et al., 1998 for excellent reviews). Ire1 and PERK are normally kept in an inactive state though an association between their N-terminal lumenal domains and the chaperone BiP. Under conditions of ER stress, BiP dissociates (to bind unfolded proteins) and Ire1 and PERK undergo homo-oligomerization, stimulating trans-autophosphorylation within their serine/threonine kinase domains. Ire1 also contains a C-terminal endonuclease domain that excises a short sequence from the mRNA of the X-Box binding protein (XBP-1), generating an active bZIP transcription factor that

stimulates transcription of ER chaperone genes (Calfon et al., 2002; Ma and Hendershot, 2001; Shen et al., 2001; Yoshida et al., 2001). PERK, on the other hand, phosphorylates the translation initiation factor eIF2α, which halts translation and prevents the continuing accumulation of newly synthesized proteins into the ER when protein folding conditions are compromised (Harding, *et al.*, 1999). Upregulation of ER chaperone genes is also mediated by a second, perhaps redundant, pathway involving ATF6. In this case, ATF6 undergoes proteolytic cleavage at the ER, which releases its active bZIP transcription factor domain to the nucleus (Ye, et al., 2000; Yoshida, et al., 1998).

1.8.1.2 ER Stress: the Death Response

Experimentally, ER stress is induced by pharmacological agents that inhibit N-linked glycosylation (tunicamycin, TN), block ER to Golgi transport (brefeldin A, BFA), impair disulfide bond formation (dithiothreitol, DTT), or disrupt ER Ca^{2+} stores (thapsigargin, TG, an inhibitor of the sacroplasmic/endoplasmic reticulum Ca^{2+} ATPase [SERCA] pumps, or A-23187, a Ca^{2+} ionophore). All of these agents eventually induce apoptosis within 20-48 hrs depending on the cell type, suggesting that if the damage to the ER is too great, or if balance is not restored within a certain window of time, an apoptotic response is elicited (Patil and Walter, 2001). The mechanism by which ER stress is coupled to activation of caspases was for the most part a mystery until caspase-12 was characterized by Nakagawa and Yuan. Caspase-12 is ubiquitously expressed and, like all caspases, synthesized as an inactive proenzyme consisting of a regulatory prodomain and two catalytic p20 and p10 subunits (Nakagawa and Yuan, 2000; Van de Craen et al., 1997). However, unlike other caspases, caspase-12 is remarkably specific to

insults that elicit ER stress and is not proteolytically activated by other death stimuli (Nakagawa et al., 2000). Accordingly, *caspase-12*-null mice and cells are partially resistant to apoptosis induced by ER stress but not by other apoptotic stimuli. (Nakagawa et al., 2000).

Caspase-12 is localized at the cytosolic face of the ER, placing it in a position to respond to ER stress as a proximal signaling molecule (Nakagawa and Yuan, 2000). However, the mechanism of caspase-12 activation is unclear. Nakagawa and Yuan showed that in mouse glial cells undergoing ER stress caused by oxygen and glucose deprivation, caspase-12 was cleaved by calpain (Nakagawa and Yuan, 2000). In vitro, m-calpain cleaved caspase-12 at T132 and K158, which releases the prodomain from the catalytic subunits and increases enzymatic activity (Nakagawa and Yuan, 2000). It has also been suggested that caspase-12 activation is linked to Ire1 signaling. The cytosolic tail of Ire1 can recruit TRAF2 (Urano et al., 2000) and when overexpressed, TRAF2 can interact with caspase-12 and weakly induce its oligomerization and cleavage (Yoneda et al., 2001). Moreover, Ire1 induces apoptosis when overexpressed (Wang et al., 1998), which could be due to caspase-12 activation. The CARD in caspase-12's prodomain might mediate homotypic interaction with other CARD containing proteins, allowing its recruitment into such an activation complex (analogous to caspase-9 recruitment to Apaf-1), or mediate caspase-12 oligomerization and autoactivation at the ER. Overexpression of full-length caspase-12 induces its oligomerization and self cleavage between the p20 and p10 subunits at D318 (Fujita et al., 2002; Nakagawa et al., 2000; Rao et al., 2001), but only caspase-12 lacking its prodomain induces apoptosis. Therefore, caspase-12 activation likely involves both calpain-dependent removal of the prodomain and self-cleavage at D318.

Following its activation at the ER, caspase-12 may directly process downstream caspases in the cytosol or target other as yet unidentified substrates that influence the progression of apoptosis. Two groups recently reported that caspase-12 can directly cleave procaspase-9 in vitro, leading to caspase-9 dependent activation of caspase-3 (Morishima et al., 2002; Rao et al., 2002). Inhibition of caspase-12 by expression of MAGE-3, a protein that binds the p10 subunit and blocks catalytic activity, prevents TG- and TN-induced processing of caspase-9 and -3 (Morishima et al., 2002). In addition, ER stress-induced processing of procaspase-9 can occur in the absence of cyt.c release and in *APAF-1*-null fibroblast (Rao et al., 2002). These results argue that caspase-12 can directly trigger caspase-9 activation and apoptosis independent of the mitochondrial cyt.c/Apaf-1 pathway, at least in certain cell types. Many studies, however, implicate the involvement of mitochondria in ER stress induced apoptosis (below), which might represent a redundant pathway to caspase activation or, alternatively, might provide a pathway for accumulating Smac/Diablo and HtrA2 in the cytosol, where their inhibition of IAPs supports optimal activation of caspases.

The relevance of caspase-12 signaling has been somewhat clouded by the fact that a human ortholog remains elusive. Fischer et al. reported that the human caspase-12 gene has acquired deleterious mutations that prevent the expression of a functional protein (Fischer et al., 2002). In spite of this discovery, two reports showed that antibodies against murine caspase-12 detect an appropriately sized protein in human cells that is processed following ER stress (Nakagawa et al., 2000; Rao et al., 2001). Proof that a functional human caspase-12 protein does indeed exist, therefore, must await purification of the endogenous enzyme.

1.8.1.3 ER Stress-Induced Death: Contribution of Mitochondria

Several lines of evidence suggest that mitochondria are an important component of the ER stress-induced apoptotic pathway. First, ER stress agents cause mitochondrial release of cyt.c and loss of mitochondrial transmembrane potential (Boya et al., 2002; Hacki et al., 2000); second, Bcl-2/Bcl-X_L inhibit ER stress-induced apoptosis (McCormick, *et al.*, 1997, McCullough, *et al.*, 2001; Hacki et al)); and third, *Bax-/-, Bak-/-* MEFs are resistant to TG- , TN-, and BFA-induced apoptosis (Wei et al., 2001). While the latter two findings could be due to potential roles of Bcl-2 family members at the ER (see below), Kroemer and colleagues confirmed the involvement of mitochondria by showing that the cytomegalovirus encoded mitochondrial inhibitor of apoptosis (vMIA) potently inhibited ER stress apoptosis (Boya et al., 2002). Signaling between the ER and mitochondria presumably involves the activation of one or more BH3-only proteins. BAD is a candidate, since TG or A23187 have been shown to induce its Ca^{2+}/calcineurin-dependent dephosphorylation and activation (Wang et al., 1999). Increased cytosolic ($[Ca^{2+}]_c$) has also been observed following other conditions of ER stress (Carlberg et al., 1996) and, therefore, BAD activation may be a conserved component of the pathway. Additionally, the UPR might transcriptionally upregulate other BH3-only proteins, analogous to the response of BH3-only genes to p53-mediated stress responses.

ER stress-induced cyt.c release is apparently dependent on the c-Abl tyrosine kinase, because c-Abl-/- mouse embryonic fibroblasts are resistant to A23187-, BFA-, and TN-induced cyt.c release and apoptosis (Ito et al., 2001). c-Abl redistributes from the ER to the mitochondria within several hours of TG application, which parallels an increase in its kinase activity. The mechanism by which c-Abl exerts its action at this site is unclear, but it may function in concert with JNK kinases, which are recruited and

activated by Ire1 during ER stress (Urano, *et al.*, 2000), and are essential for mediating cyt.c release in other cell death pathways (Tournier et al., 2000). UPR upregulation of CHOP/GADD153, a nuclear transcription factor that represses the Bcl-2 promoter (McCullough et al., 2001), may sensitize mitochondria to the proapoptotic effects of BH3-only proteins by decreasing the cellular levels of Bcl-2 protein. Consistent with this, tunicamycin-induced apoptosis is impaired in CHOP -/- MEFs, and CHOP -/- mice injected with tunicamycin show decreased apoptosis in the renal tubular epithelium (Zinszner et al., 1998).

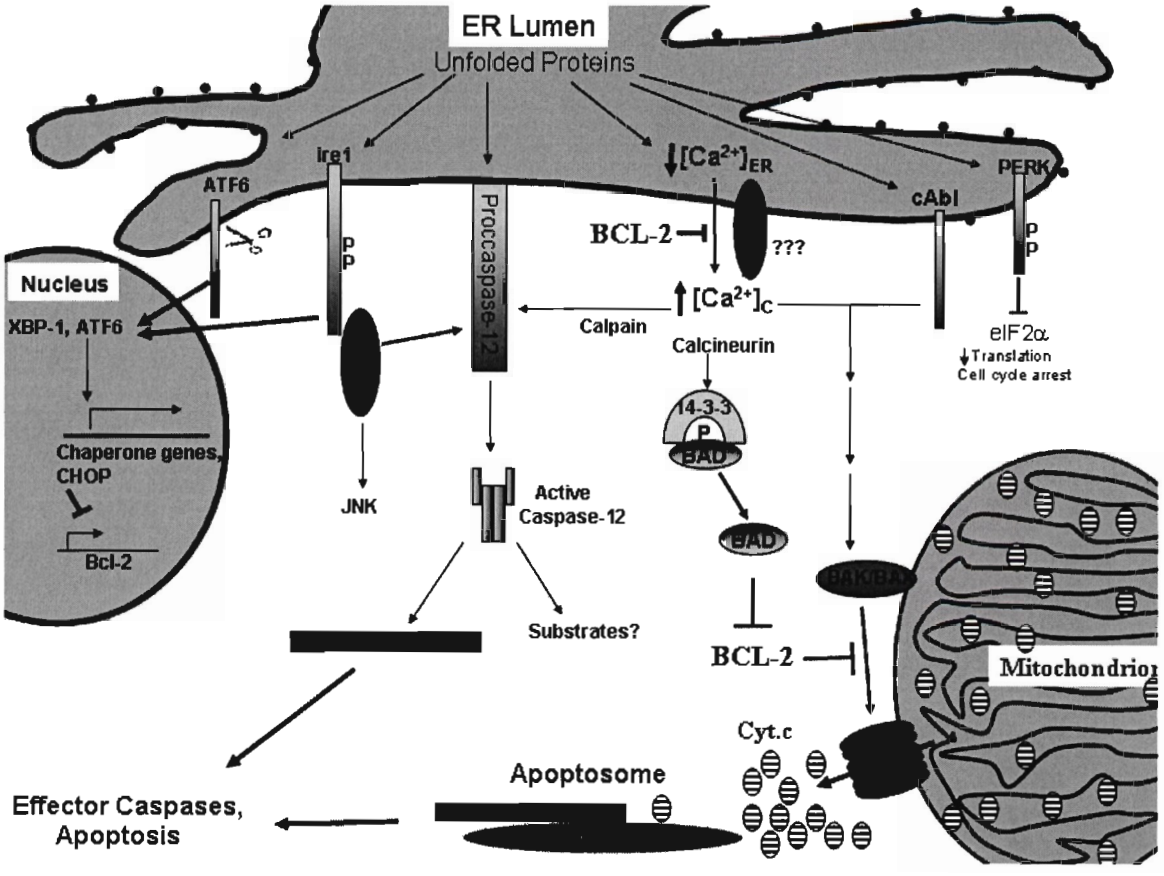

Figure 1.5. ER stress signaling pathways. See text for details.

Therefore, stress in the ER unleashes a mitochondrial-dependent apoptotic pathway and a caspase-12, mitochondrial-independent pathway (Fig. 1.5). These two arms of the ER stress response apparently operate independently of one another, since zVAD-fmk, which effectively inhibits caspase-12 in vitro (Nakagawa and Yuan, 2000), has no effect on ER stress-induced cyt.c release (Hacki et al., 2000), and caspase-12 -/- MEFs only partially resist apoptosis (Nakagawa et al., 2000). This duality in signaling may ensure complete and efficient caspase activation in physiological settings.

1.8.2 Regulation of Apoptosis by ER Ca^{2+} Signals

The ER, or its equivalent in muscle cells, the sacroplasmic reticulum (SR), represents the cell's largest calcium store. SERCA pumps located in the ER membrane maintain the $[Ca^{2+}]_{ER}$ up to three orders of magnitude higher than the $[Ca^{2+}]_c$ (Pozzan et al., 1994). The release of Ca^{2+} from the ER is primarily achieved by two well characterized types of channels, the inositol 1, 4, 5-triphosphate receptor (IP_3R) and the Ryanodine receptor (RyR) families (Berridge et al., 2000; Pozzan et al., 1994). As well, other Ca^{2+} release channels likely exist (Cavalli et al., 2002). Release of calcium from the ER has been observed in many forms of apoptosis and regulated oscillations of Ca^{2+} levels by IP_3R- mediated spikes may directly regulate the cell death machinery. Jurkat cells deficient in type 1 IP_3R (IP_3R1) show smaller increases in $[Ca^{2+}]_c$ in response to dexamethasone, ionizing radation, and T cell receptor or Fas stimulation, and resist subsequent apoptosis (Jayaraman and Marks, 1997). Moreover, targeted disruption of all three IP_3R isoforms in the chick DT40 B-cell line blocked Ca^{2+} mobilization and apoptosis following B-cell receptor crosslinking. The degree of resistance increased with

the number of IP_3R genes deleted, suggesting that all three isoforms can contribute to apoptotic signaling (Sugawara et al., 1997). How IP_3R activation is coupled to such diverse death signals, however, remains to be determined.

The way in which Ca^{2+} signals engage cell death pathways may largely be decided by the spatio-temporal pattern and intensity of ER Ca^{2+} release. IP_3R/RyR dependent global increases in $[Ca^{2+}]_c$ can influence apoptosis by several mechanisms. For example, Ca^{2+} dependent activation of calpains has been implicated in activating caspases in several forms of apoptosis (Wang, 2000), including ER stress (Nakagawa and Yuan, 2000), B-cell receptor-, (Ruiz-Vela et al., 1999; Ruiz-Vela et al., 2001), and radiation-induced apoptosis (Waterhouse et al., 1998). In addition, calpain cleavage of BAX has been reported to increase its proapoptotic activity (Wood et al., 1998), and in some cases calpain may cooperate with caspases in the execution phase of apoptosis (Wood and Newcomb, 1999). As discussed above, Ca^{2+} signals can lead to activation of BAD, and Ca^{2+} dependent activation of the transcription factor MEF2 leads to upregulation of Nur77/TR3 (Youn et al., 1999), which can bind to mitochondria and induce cyt.c release (Li et al., 2000). In contrast, privileged transport of Ca^{2+} between juxtaposed ER and mitochondrial membranes may sensitize mitochondria to the effects of proapoptotic BCL-2 family members (Hajnoczky et al., 2000a), perhaps through Ca^{2+}-induced opening of the mitochondrial permeability transition pore.

1.8.2.1 Ca^{2+} Signaling Between the ER and Mitochondria

Mitochondria have the capacity to accumulate high Ca^{2+} loads and Ca^{2+} shuttling between the ER and mitochondria regulates normal physiological processes including, oxidative respiration (Rizzuto et al., 2000). Ca^{2+} can freely diffuse across the OMM,

transport across the impermeable inner mitochondrial membrane (IMM) is facilitated by a mitochondrial membrane potential ($\Delta\Psi_m$) driven, low affinity uniporter, that is activated by elevated $[Ca^{2+}]_c$ (see Bernardi, 1999, Crompton, 1999, Duchen, 2000 for reviews). Calcium efflux from the mitochondria occurs through a Na^+-Ca^{2+} exchanger and also by Na^+-independent mechanism (likely a H^+-Ca^{2+} exchanger). Simultaneous measurement of $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ has revealed that following activation of the IP_3R or RyR, cytosolic Ca^{2+} spikes are simultaneously accompanied by parallel spikes in $[Ca^{2+}]_m$, highlighting the close coupling of ER Ca^{2+} release with mitochondrial uptake (Rizzuto et al., 1998). However, IP_3R and RyR channels only rise global $[Ca^{2+}]_c$ from ~100nm to ~1μM; an increase in $[Ca^{2+}]_c$ insufficient to activate the mitochondrial Ca^{2+} uniporter (Hajnoczky et al., 2000b). This issue has been resolved by a model accounting for privileged mitochondrial uptake sites that sense high local $[Ca^{2+}]_c$ in the proximity of ER Ca^{2+} release sites. Close contacts between the ER and mitochondria are clearly visible by thin section electron microscopy and in living cells mitochondria exist as a highly interconnected tubular network that is intimately associated with the ER network. Using live visualization of ER and mitochondria in HeLa cells transfected with appropriately targeted GFP constructs, Rizzuto et al., estimated that 5-20% of mitochondria are in close apposition with the ER (Rizzuto et al., 1998). Furthermore, IP_3Rs and RyRs are not homogenously distributed throughout the ER but concentrated within subdomains highly active in Ca^{2+} release, which are often in close apposition and facing mitochondrial surfaces (Mignery et al., 1989; Ramesh et al., 1998; Satoh et al., 1990; Sharma et al., 2000; Takei et al., 1992) . It has been estimated that in some cell types, coordinated Ca^{2+} release by clusters of IP_3Rs or RyRs, may elevate local $[Ca^{2+}]_c$ at ER-mitochondrial

junctions as high as 20-50 μM (> 20 fold higher than global $[Ca^{2+}]_c$ rises), facilitating rapid uptake by the mitochondrial uniporter (Hajnoczky et al., 2000b) .

1.8.2.2 Regulation of Mitochondrial Permeability Transition by ER Ca^{2+} Spikes during Apoptosis

The high $[Ca^{2+}]_m$ that follows IP_3R/RyR spikes has recently been linked to mitochondrial release of apoptogenic factors through the opening of the permeability transition pore (PTP). It has been known for many years that mitochondrial Ca^{2+} overload is a strong inducer of PTP opening in isolated mitochondria (Crompton, 1999). Elegant studies by Hajnoczky and coworkers have recently demonstrated that ER Ca^{2+} spikes can facilitate PTP activation during apoptosis in vivo (Szalai et al., 1999). In intact or permeablized cells high mitochondrial Ca^{2+} loads generated by IP_3R stimulation or exogenous Ca^{2+} spikes lead to weak PTP activation, characterized by small, transient mitochondrial depolarizations that are reversible and that do not release apoptotic proteins from the IMS. So called "PTP flickering" may be a normal process in mitochondrial physiology (Crompton, 1999; Duchen, 2000). However, if cells are briefly challenged with apoptotic agents that act on mitochondria, such as C2 ceramide or staurosporin, mitochondria become sensitized to IP_3R-induced Ca^{2+} spikes resulting in complete mitochondrial depolarization and release of cyt.c. Inhibition of the mitochondrial Ca^{2+} uniporter with RuRed or addition of the PTP inhibitor CsA completely prevented both loss of $\Delta\Psi_m$ and cyt.c release, suggesting that these events are dependent on mitochondrial Ca^{2+} uptake followed by PTP opening. Intriguingly, if the cells were subsequently washed to remove the apoptotic stimulus and the IP_3 inducing agonist

(ATP), $\Delta\Psi_m$ fully recovered but the cells eventually underwent apoptosis. These results suggest that during apoptosis IP_3R driven Ca^{2+} spikes may cooperate with other mitochondrial apoptotic signals to induce transient opening of the PTP, facilitating release of IMS apoptotic agents into the cytosol, followed by resealing of the PTP and regeneration $\Delta\Psi_m$, allowing continued production of ATP for the execution phase of apoptosis (Szalai et al., 1999). Of note, however, in vivo cyt.c release normally occurs in the absence of obvious mitochondrial depolarizations (Goldstein et al., 2000). Therefore, in a physiological context suboptimal ER Ca^{2+} spikes might lead to transient collapses in $\Delta\Psi_m$ that are important for IMM cristae remodeling.

How IP_3R/RyR Ca^{2+} release channels are activated during apoptosis, and whether these receptors actually induce Ca^{2+} spikes that are received by mitochondria during physiological forms of apoptosis remains to be demonstrated. Nonetheless, several other independent studies suggest that Ca^{2+} signals between the ER and mitochondria are important in diverse cell death pathways. For instance, apoptosis induced by staurosporin in neural cells (Kruman and Mattson, 1999), C6 ceramide in U937 cells (Quillet-Mary et al., 1997), glucocorticoid stimulation of lymphocytes, activation-induced death of T cell hybridomas, and TNF induced death of U937cells (Zamzami et al., 1995) are all blocked by inhibition of mitochondrial Ca^{2+} uptake. In addition, changes in ER Ca^{2+} homeostasis have been shown to affect the sensitivity of cells to apoptosis. Stable overexpression of calreticulin, a major Ca^{2+} binding ER chaperone, increases the ER Ca^{2+} storage and enhances agonist induced IP_3R Ca^{2+} release, resulting in increased cyt.c release, caspase activation, Annexin V staining and tunnel reactivity in response to thapsigargin, staurosporin, and etoposide (Nakamura et al., 2000). In contrast, *calreticulin*-null MEFs are defective in IP_3R-dependent Ca^{2+} efflux (Mesaeli et al., 1999), and resist etoposide,

staurosporin and UVB induced apoptosis, and show decreased cyt.c release and caspase activation (Nakamura et al., 2000). Moreover, overexpression of BCL-2 alters ER Ca^{2+} homeostasis and BCL-2 targeted exclusively to the ER protects cells against many forms of apoptosis (see below). Therefore, IP_3R/RyR mediated spikes in $[Ca^{2+}]_m$ may participate in the mitochondrial phase of apoptosis in many systems. It is important to emphasize, however, that at present the data suggest a role for ER Ca^{2+} signals in sensitizing mitochondria to respond to other apoptotic effectors in certain pathways rather than functioning as an obligate initiation event.

1.8.3 Bcl-2 Family Proteins and the ER

1.8.3.1 Bcl-2 Family Proteins and the ER: Anti-Apoptotic Members

Although the function of Bcl-2 proteins is best characterized at the mitochondrion, these proteins also localize to other intracellular membranes such as the ER / nuclear envelope. Studies employing BCL-2 selectively targeted to the ER with the transmembrane sequence of cytochrome b5 (b5-BCL-2) have demonstrated that BCL-2 has antiapoptotic activity at this location. For example, b5-BCL-2 prevents apoptosis induced by ER stress agents, ceramide, Myc, ionizing radiation, or BAX overexpression (Annis et al., 2001; Hacki et al., 2000; Rudner et al., 2001; Wang et al., 2001b) . Annis et al. observed that b5-BCL-2 conferred protection against apoptotic stimuli that caused an obvious mitochondrial depolarization prior to the release of cyt.c (Myc, C2 ceramide), but not against stimuli that induced cyt.c release in the absence of large mitochondrial depolarizations (etoposide) (Annis et al., 2001). Wild type BCL-2, in contrast, could inhibit both types of stimuli. The fact that ER-localized BCL-2 can only inhibit certain apoptotic pathways suggests that its antiapoptotic effect at the ER may not be the result of

simple sequestration of endogenous pro-apoptotic Bcl-2 proteins, in which case its membrane location would be irrelevant. This assumes, however, that the various pathways tested are indeed coupled to pro-apoptotic BCL-2 binding partners.

Both pro- and anti-apoptotic Bcl-2 proteins have pore forming properties (Antonsson and Martinou, 2000) and, in theory, could influence ion flux across cellular membranes. Not surprisingly, therefore, many studies implicate BCL-2 in regulating ER Ca^{2+} homeostasis. However, the mechanism by which it does so is controversial and may depend on the cell type tested. For example, several groups have reported that BCL-2 increases the ER Ca^{2+} content and/or prevents ER Ca^{2+} release during apoptosis (Distelhorst, *et al.*, 1996, He, *et al.*, 1997, Ichimiya, *et al.*, 1998, Lam, *et al.*, 1994, Nutt et al., 2002). In contrast, studies by Rizzuto and colleagues convincingly demonstrated that overexpression of BCL-2 reduces the steady state level of $[Ca^{2+}]_{ER}$ by increasing the permeability of the ER membrane to Ca^{2+} (Pinton, *et al.*, 2000). Consistent with this latter finding, there are now several examples in the literature where manipulations that lead to decreased $[Ca^{2+}]_{ER}$ protected cells against apoptosis, whereas manipulations that increased $[Ca^{2+}]_{ER}$ sensitized cells to apoptosis (Lilliehook et al., 2002; Nakamura et al., 2000; Pinton et al., 2001). Changes in $[Ca^{2+}]_{ER}$ affect the intensity IP_3R/RyR Ca^{2+} spikes, which are known to sensitize mitochondria to BH3-dependent cyt.c release during apoptosis (Csordas et al., 2002). It has also been reported that BCL-2 can increase the capacity of mitochondria to store Ca^{2+} (Ichimiya et al., 1998; Murphy et al., 1996; Zhu et al., 1999), presumably by preventing the opening of the PTP, which releases matrix Ca^{2+}. Thus, by localizing to both ER and mitochondria, BCL-2 might prevent apoptotic cross talk between the two compartments by lowering the amount of free $[Ca^{2+}]_{ER}$ for IP_3R/RyR release and by increasing the tolerance of mitochondria to high Ca^{2+} loads.

1.8.3.2 Bcl-2 Family Proteins and the ER: Proapoptotic BAX and BAK

The pro-apoptotic multidomain BCL-2 family members BAX and BAK have also been reported to localize to the ER in some cell types (Nutt et al., 2001) and these proteins may induce apoptosis from this location. For example, when over-expressed in human PC-3 cells, BAX and BAK localize to both the mitochondria and ER, and induce caspase-independent emptying of ER Ca^{2+} pools concomitant with an increase in mitochondrial Ca^{2+} pools (Nutt et al., 2001; Pan et al., 2001). Co-expression of BCL-2/BCL-xL inhibits this Ca^{2+} mobilization, and an inhibitor of mitochondrial Ca^{2+} uptake, RU360, blocked BAX/BAK- and staurosporin-induced increase in $[Ca^{2+}]_m$, and subsequent cyt.c release and apoptosis (Nutt et al., 2001). Moreover, BAX-null DU-145 cells resist staurosporin-induced ER Ca^{2+} release, mitochondrial Ca^{2+} uptake and cyt.c release, all of which are overcome by re-expression of BAX (Nutt et al., 2002). Consistent with a role for these proteins at the ER, BAK and BAX interact with the cytosolic tail of the ER chaperone calnexin in yeast, and BAK induced lethality in *S. pombe* is dependent upon this interaction (Torgler et al., 1997). Moreover, b5-BCL-2 can inhibit Bax induced apoptosis (Wang et al., 2001b) as can BAX inhibitor-1, an antiapoptotic transmembrane protein of the ER (Xu and Reed, 1998). BAX and BAK may have dual roles at the ER and mitochondria and help facilitate cross-talk between the two organelles or, conversely, BAX and BAK may activate discrete death pathways at the ER and mitochondria. Consistent with the latter hypothesis, *Bax,Bak* double-null cells are completely resistant to diverse apoptotic stimuli, whereas cells lacking components of the mitochondrial apoptosome apparatus (cyt.c, Apaf-1, or caspase-9) only partially resist apoptosis. It must be stressed, however, that the observed effects of Bcl-2 proteins on ER Ca^{2+} homeostasis could be indirect and secondary to their effects on mitochondria. BCL-

xL, for example, was shown to modulate ER Ca^{2+} homeostasis through its effects on oxidative phosphorylation at the mitochondria, which lowers NFAT-dependent transcription of the *IP_3R* gene (Li et al., 2002a). These questions may require targeted localization of ectopic BAX or BAK in otherwise *Bax,Bak* double-null cells.

1.8.3.3 Bcl-2 Family Proteins and the ER: BH3-only Members

Recent models hold that the BH3-only subset of proapoptotic Bcl-2 family members sense diverse death signals and respond by initiating caspase activation (Puthalakath and Strasser, 2002). BH3-only molecules may achieve this by binding and inhibiting anti-apoptotic Bcl-2 family members or by directly activating proapoptotic BAX and BAX (Letai et al., 2002a). Certain BH3-only molecules, after activation, translocate to mitochondria and induce the oligomerization of BAX and BAX into predicted pores in the OMM, facilitating egress of the protein contents of the intermembrane space. Consistent with this model, most BH3-only proteins localize to mitochondria, at least in part, and trigger cyt.c release, which in all cases is inhibited by BCL-2 overexpression.

Given that representatives from all three groups of the BCL-2 family have been found at the ER, however, it is likely that the ratiometric interactions between anti-apoptotic and pro-apoptotic members that regulate the mitochondrial apoptotic pathway also regulate apoptotic pathways at the ER (Figure 2). The BH3-only protein BIK, for example, is located primarily at the ER (Mathai et al., 2002). BIK mRNA and protein is induced by p53 in response to DNA damage or oncogenic stress (Mathai et al., 2002; unpublished), and ER BIK induces cyt.c release independent of an association with mitochondria or zVAD-sensitive caspases (Germain et al., 2002). An S9 fraction containing both microsomes and cytosol, but not either fraction alone, isolated from BIK-

treated H1299 cells (in the presence of caspase inhibitors) can induce mitochondrial release of cyt.c in vitro. This suggests that BIK activates factors in both the ER and cytosol to induce mitochondrial transformations (Germain et al., 2002). This activity of BIK is influenced by BCL-2 at the ER since BIK/BCL-2 heterodimers can be crosslinked at the surface of the ER and high BIK:BCL-2 ratios change the set of proteins BCL-2 interacts with at the ER, resulting in Ca^{2+} release, egress of cyt.c from mitochondria, and apoptosis (M.Germain and G. Shore, unpublished). When in excess, however, ER-localized BCL-2 is able to protect against BIK-induced apoptosis. The ratio of BH3-only and anti-apoptotic BCL-2 family members at the ER, therefore, may regulate downstream signals emitted from the organelle, including Ca^{2+} signals and/or proximal zVAD-insensitive caspase activation, which in turn regulate mitochondrial release of cyt.c. Other BH3-only proteins, such as BIM and NOXA (Oda et al., 2000; Puthalakath et al., 1999), also partially locate to the ER and, therefore, may elicit similar effects.

By analogy to their known functions at mitochondria, it is also possible that certain BH3-only proteins induce BAX/BAK oligomerization in the ER membrane and mediate release of lumenal proteins that regulate caspase activation. Additionally, BAX/BAK pores might control ER Ca^{2+} stores, influencing local $[Ca^{2+}]_c$ and/or facilitating mitochondrial Ca^{2+} uptake and sensitization for cyt.c release. This model is consistent with pore forming properties of Bcl-2 proteins, their ability to modulate movement of Ca^{2+} across the ER membrane, and the dependence of most BH3-only proteins on BAX/BAK to induce apoptosis (Cheng et al., 2001). Moreover, the ER undergoes profound dilation and unfolding of cisternae during apoptosis (Johnson et., al 2000; Chang, *et al.*, 2000, Herrera, *et al.*, 2000, Muriel, *et al.*, 2000, Weller, *et al.*, 1995,

Wu, *et al.*, 1999, Zeng and Xu, 2000) suggesting that ER homeostasis and volume control is lost, perhaps due to membrane permeabilization.

1.8.4 Regulation of Initiator Caspases at the ER by Bcl-2 Proteins

Other apoptotic models posit that Bcl-2 proteins regulate caspase activation upstream or in parallel to the mitochondrial pathway (Hsuang and Stasser., 2000; Marsden et al., 2002) and that mitochondria simply amplify weak initiator caspase signals. As discussed above, caspase-12 activation at the ER membrane clearly represents one mechanism of mitochondria-independent initiator caspase activation. The ability of ER-localized Bcl-2 family members to directly regulate this caspase certainly deserves testing. Additionally, the novel procaspase-8 isoform, procaspase-8L, like procaspase-12, is peripherally associated with the cytosolic face of the ER (See Chapter 2; Breckenridge et al., 2002). However, procaspase-8L processing seems to be regulated through an association with the BAP31 complex. BAP31 is a ubiquitously expressed polytopic integral membrane protein of the ER that associates with related BAP29, components of the actomyosin network, and BCL-2/BCL-xL (Adachi et al., 1996; Ng et al., 1997; Nguyen et al., 2000). Procaspase-8L, but not procaspase-8, is selectively recruited to the BAP31 complex during oncogenic E1A-induced apoptosis, which coincides with its proteolytic processing. This recruitment is dependent on procaspase-8L's Nex domain, a 59 amino acid stretch that extends canonical procaspase-8/a at the N-terminus. E1A-induced processing (and presumably activation) of procaspase-8L is regulated by its association with BAP proteins since cells doubly deficient in Bap31 and Bap29 do not process procaspase-8L in response to E1A and show reduced caspase-8 and -3 activity and subsequent apoptosis. It is possible that the cytosolic tails of BAP31 and

BAP29 recruit factors that facilitate procaspase-8L activation. Importantly, BCL-2 overexpression does not inhibit recruitment of procaspase-8L to the BAP31 complex but abrogates subsequent enzyme processing and apoptosis. Moreover, E1A-induced processing of procaspase-8L is subject to ratiometric regulation by BIK and Bcl-2 located at the ER (unpublished), suggesting that it might be a target of BH3-initiated caspase activation. At present it is not known whether other initiator caspases respond to signals at the ER. Given that initiator procaspase-2 has been implicated upstream of mitochondrial dysfunction during drug induced apoptosis (Lassus et al., 2002) and that this caspase is located at Golgi and ER membranes in some cell types (Mancini et al., 2000), however, it is conceivable that procaspase-2 is also regulated by Bcl-2 proteins at the ER.

The massive surface area of the ER membrane may provide a platform for the assembly of caspase regulatory complexes. This model parallels apoptotic regulation in *C. elegans*, wherein a Bcl-2 protein (CED-9) binds an adapter molecule (CED-4) preventing it from activating a caspase (CED-3). By binding CED-9, BH3-only EGL-1 initiates apoptosis by displacing CED-4 (Metzstein et al., 1998). BCL-2 can compensate for *ced-9* loss-of-function mutations in nematodes, indicating that BCL-2 may be able to function in a similar manner in mammalian cells (Hengartner and Horvitz, 1994). For example, upregulation of BIK following oncogenic stress increases the BIK:BCL-2 ratio at the ER, which might relieve the inhibitory effect of BCL-2 on procaspase-8L processing. ER proteins such as the reticulon family members NSP-C and RTN-x_s, which bind and inhibit BCL-2 and BCL-xL (Tagami et al., 2000), or p53-inducible genes that target the ER, including PIDD and Scotin (Bourdon et al., 2002; Lin et al., 2000), might also play a role in the regulation of caspase activation at the ER. Alternatively, high

BH3/BCL-2 ratios may relieve BCL-2 inhibition of some other apoptotic process at the ER, notably calcium homeostasis.

The various models for the function of BCL-2 family proteins at the ER are not mutually exclusive and one could imagine that, depending on the apoptotic stimulus, multiple signaling pathways could emerge from the ER to either directly activate the mitochondrial pathway (as with BIK) via caspase-dependent and –independent means, or to sensitize mitochondria to a secondary direct BH3 hit at this organelle (as with ER Ca^{2+}

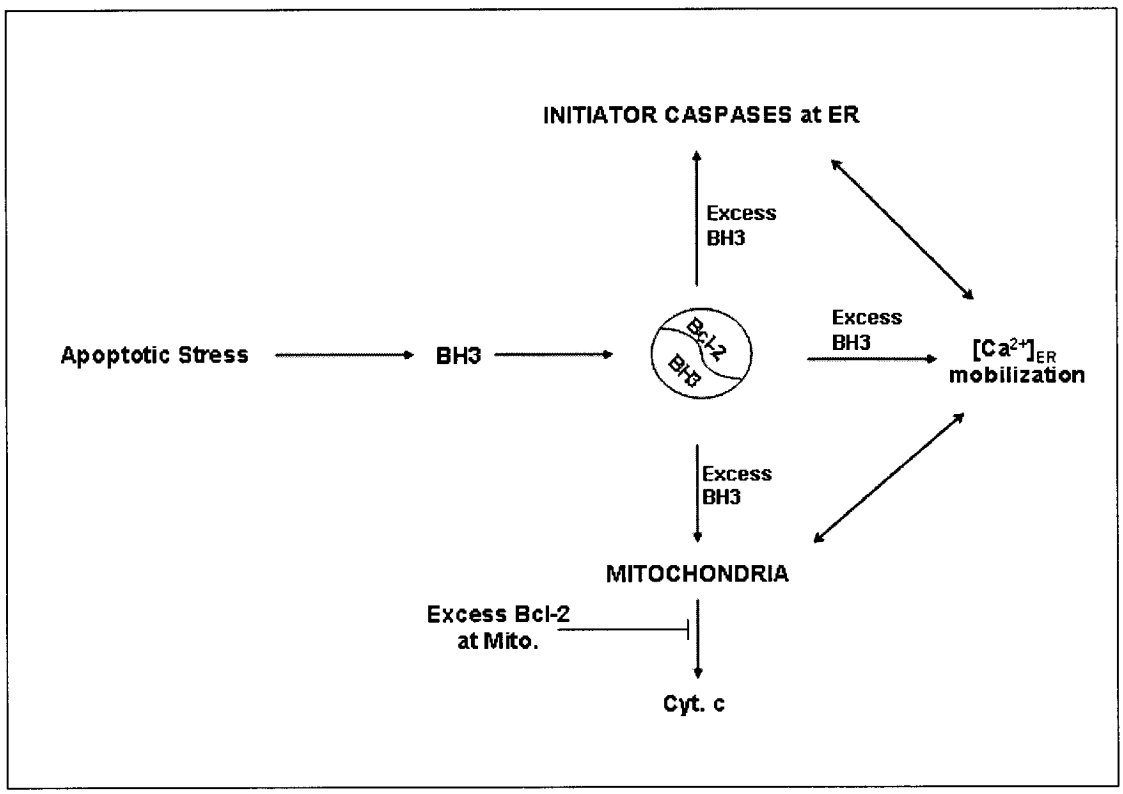

Figure 1.6. BH3-driven apoptotic pathways at the ER. Diverse apoptotic stresses may increase the concentration of BH3-only proteins, such as BIK, at the ER. When in excess, BH3-only proteins may mobilize ER Ca^{2+} stores and sensitize mitochondria to undergo apoptotic transitions in response to a direct BH3 hit, or activate other Ca^{2+}-dependent processes in the cytosol. BH3-only proteins might also stimulate other Ca^{2+}-indpendent pathways leading to cyt.c release from the mitochondria or facilitate local procaspase-12, procaspase-8L, or procaspase-2 activation at the ER surface. To accomplish these tasks, BH3-only proteins may simply relieve BCL-2 repression of these processes or, additionally, they may play a second role in activating these pathways after BCL-2 is repressed. It remains to be determined if the ratiometric influence of anti- and pro-apoptotic members also includes BAX,BAK.

release), or to synergize with mitochondrial cyt.c release to activate downstream caspases (as with the caspase-12/ER stress pathway) (Fig. 1.6).

1.8.5 Caspase substrates located at the ER

Several ER proteins have now been identified as caspase substrates. The majority seem to be caspase-3 targets and, therefore, their cleavage likely contributes to the coordinated shutdown of normal cellular processes during the execution phase of apoptosis.

1.8.5.1 BAP31

An exception, however, is BAP31, which in addition to being a regulator of procaspase-8L, is also a caspase-8 substrate itself (Ng et al., 1997). The cytosolic tail of human BAP31 contains two identical caspase recognition sequences that are rapidly cleaved by caspase-8 following stimulation of the Fas death receptor (Nguyen et al., 2000; Wang et al., 2003), and presumably following procaspase-8L activation at the ER (Breckenridge et al., 2002). Fas initiates apoptosis by directly recruiting and activating procaspase-8 at the plasma membrane. Caspase-8 in turn cleaves the BH3-only molecule, BID, generating tBID, which induces BAX/BAK-dependent cyt.c release from mitochondria and subsequent activation of downstream caspases (Korsmeyer et al., 2000). In some cell types, Fas signaling does not require the participation of mitochondria for apoptotic execution because sufficient caspase-8 is activated to permit direct cleavage and activation of downstream caspases (Scaffidi et al., 1998). Stable expression of a caspase-resistant BAP31 (crBAP31) mutant, in which the caspase-8

recognition asp residues have been substituted with ala, strongly inhibits Fas induced apoptosis in KB epithelial cells (see Chapter 3; Nguyen et al., 2000). While crBAP31 had a very weak influence on Fas-induced caspase activation or cleavage of effector caspase substrates, it strongly inhibited cyt.c release, mitochondrial depolarization, cytoskeletal reorganization, and membrane blebbing. The fact that crBAP31 inhibits these events in the face of caspase activation suggests that, at least in these intact epithelial cells, the ER exerts a restraint on apoptotic mitochondrial transition and cytoplasmic restructuring that is overcome by BAP31 cleavage. Interestingly, crBAP31 did not inhibit Fas-induced cleavage of BID or downstream insertion of BAX into mitochondria, but strongly inhibited subsequent activation and oligomerization of BAX and BAK (Wang et al., 2003). To exert this anti-apoptotic effect on mitochondria, BAP31 requires an association with the putative ion channel protein of the ER, A4. Although speculative at present, it is possible that BAP31-A4 complex might regulate Ca^{2+} flux between the ER and mitochondria during Fas-mediated apoptosis, which in turn sensitizes mitochondria to tBID. Indeed, dynamic ER Ca^{2+} spikes have been implicated in mediating TNF-α-induced cyt.c release (Pu et al., 2002)

Caspase-8 cleavage of BAP31 generates a membrane embedded p20 fragment that induces cell death when expressed ectopically for prolonged periods (Ng et al., 1997). Adenoviral expression of p20 induces an immediate release of Ca^{2+} from the ER, which is followed by mitochondrial recruitment of Drp1, a critical mediator of OMM fission, and dramatic fragmentation and fission of the mitochondrial network into small punctiform organelles (See Chapter 4; Breckenridge et al., 2003). p20 seems to regulate the fission machinery through Ca^{2+} signals because inhibiting ER – mitochondria Ca^{2+} transmission

prevent Drp1 redistribution and mitochondrial fission. Interestingly, ER Ca^{2+} signals have also been shown to regulate mitochondrial restructuring during ceramide-induced apoptosis (Pinton et al., 2001). Given that Drp1-dependent mitochondrial fission is a requirement for cyt.c release during apoptosis (Frank et al., 2001), p20 might assist other caspase-8 substrates, such as tBID, to induce mitochondrial cristae restructuring (Scorrano et al., 2002) and cyt.c release during apoptosis. Accordingly, mitochondria that had undergone p20-induced fission were strongly sensitized to caspase-8-induced cyt.c release. Collectively these data are consistent with the "two hit" model described by Hajnoczky and co-workers (Szalai et al., 1999), in which an ER Ca^{2+} signal cooperates with a direct BH3-only hit on mitochondria to efficiently promote the mitochondrial release of cyt.c. Of note, crBAP31 inhibits Fas-induced ER Ca^{2+} release and mitochondrial fission (our unpublished data). These opposing effects of crBAP31 and p20 on ER Ca^{2+} homeostasis and mitochondrial integrity are independent of each other since reconstitution of crBAP31 into Bap31-null cells (which cannot generate p20) still prevents caspase-8-induced cyt.c release, and p20 still induces its proapoptotic effects in the absence of Bap31. Thus, caspase-cleavage of BAP31, like other anti-apoptotic proteins (Cheng et al., 1997; Clem et al., 1998), converts it from an inhibitor to activator of apoptosis (Fig. 1.7).

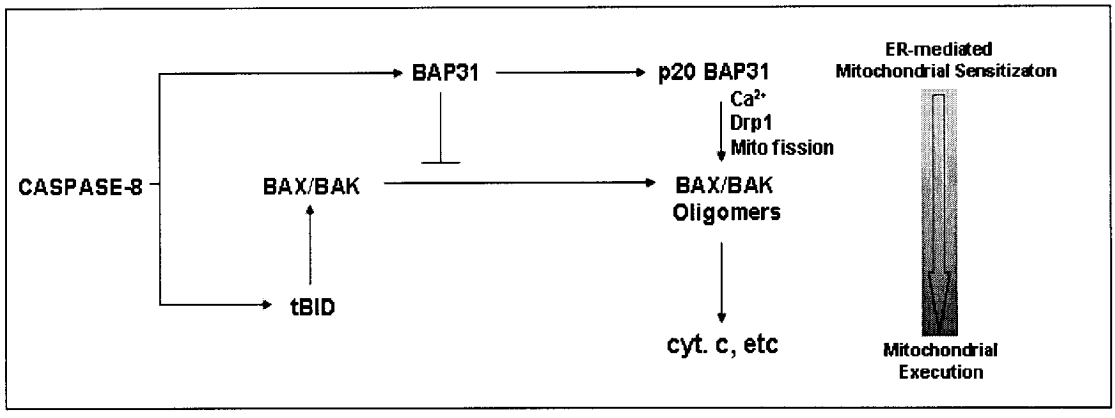

Figure 1.7. Cleavage of BAP31 at the ER sensitizes mitochondria to caspase-8-initiated release of cyt.c to the cytosol in intact cells Activation of caspase-8 leads to simultaneous processing of BID and BAP31. tBID activates cristae remodeling and BAK/BAX oligomerization in the OMM providing a conduit for cyt.c efflux across the membrane. Cleavage of BAP31 generates p20, which triggers ER Ca^{2+} release and Drp1-dependent mitochondrial fission. In contrast, full-length BAP31 prevents Fas-induced mitochondrial fission and cyt.c release. ER driven Ca^{2+} signals are likely to be modulators of apoptotic mitochondrial transition rather than obligatory events.

1.8.5.2 Other Caspase Substrates at the ER

Cleavage of other ER proteins by downstream executioner caspases might contribute to morphological progression of apoptosis or in some cases simply disable cellular processes at the ER during cell suicide. For example, caspase-3 cleavage of the sterol-regulatory element binding proteins (SREBPs) SREBP-1 and SREBP-2, upregulates sterol response genes, which may affect lipid rearrangements and/or morphological changes of the plasma membrane during apoptosis (Higgins and Ioannou, 2001). Caspase cleavage of the 72-kDa component of the signal recognition particle (SRP 72) might shutdown or alter the translation of secretory proteins during apoptosis (Keenan et al., 2001). IP_3R1 and 2 are cleaved by caspase-3 and IP_3R3 undergoes calpain proteolysis during apoptosis, which ablate IP_3-induced Ca^{2+} flux (Diaz and Bourguignon, 2000; Hirota et al., 1999). In light of the important role of IP_3R signaling during the early stages of apoptosis

(Jayaraman and Marks, 1997), caspase-mediated proteolysis might represent functional down regulation of this channel during the execution phase of apoptosis.

1.9 Summary

Apoptotic programmed cell death pathways are activated by a diverse array of cell extrinsic and intrinsic signals, most of which are ultimately coupled to the activation of effector caspases. In many instances, this involves an obligate propagation through mitochondria, causing egress of critical pro-apoptotic regulators to the cytosol. Central to the regulation of the mitochondrial checkpoint is a complex 3-way interplay between members of the Bcl-2 family, which are comprised of an anti-apoptotic sub-group including BCL-2 itself, and the pro-apoptotic BAX, BAK and BH3-domain only sub-groups. Constituents of all three of these BCL-2 classes, however, also converge on the endoplasmic reticulum (ER), an organelle whose critical contributions to apoptosis is only now becoming apparent.

In the case of ER stress, where the apoptotic signal originates within the ER itself, it is clear that the ER has its own complement of apoptotic accessories that independently activate caspases and mitochondrial dysfunction. It remains to be determined whether some of these ER signaling pathways function in other apoptotic programs. In contrast, ER Ca^{2+} signals that affect the ability of mitochondria to release IMS proteins in intact cells may be a general component of many apoptosis pathways, including the p53 and death receptor programs. It will be important to understand the nature of proapoptotic Ca^{2+} signals, how apoptotic signals converge on Ca^{2+} release channels, and how BCL-2 proteins regulate this process. Most models of cyt.c release stem from studies testing the

effect of recombinant Bcl-2 family members on isolated mitochondria (or liposomes), where the dynamic nature of the mitochondrial network and its contacts with the ER and cytoskeleton are lost. These may well impact important steps in apoptotic transformations of mitochondria in intact cells, including remodeling of cristae (Scorrano et al., 2002) and organelle fission (Frank et al., 2001). In physiological settings, the function of proapoptotic BH3-only proteins may be suboptimal and their ability to transform mitochondria into a state competent for efficient cyt.c release may largely dependent on the alliance with costimulatory signals from the ER.

A better understanding of the ER in apoptosis may have to await the clarification of BCL-2 function. Do Bcl-2 proteins operate at the ER as they do at mitochondria and influence membrane permeability? If so, and if pro-apoptotic BAX and BAK are also involved, is the mechanism different from that at the OMM (Kuwana et al., 2002)? Additionally, is ER Ca^{2+} release independently mediated by Bcl-2 proteins, IP3R/RyRs and p20 BAP31? Or do these proteins cooperate in this process? Bcl-2 proteins may also play novel roles at the ER, for example by regulating initiator caspase activation complex(es) analogous to CED-9 in *C. elegans*. Since deregulation of Bcl-2 family members at the ER affects cell survival outcomes, an understanding of their functions at this site could be important for developing new therapeutics for treating diseases such as cancer. To date, however, little has emerged in the way of elucidating the critical ER transitions during apoptosis initiation that might impact the immediate downstream pathways. In retrospect this leads one to contemplate what would our current vision of mitochondria in apoptosis be had cyt.c not been discovered as a cytosolic factor required for caspase-3 activation in vitro?

1.10 Thesis Objectives

At the time I began work on this thesis almost nothing was known about the role of the ER in apoptosis. The involvement of Ca^{2+} signaling in apoptosis, and BCL-2 function at the ER, were still hypotheses, and ER stress-induced apoptosis was merely an unexplained phenomenon. Our laboratory's discovery of ER localized BAP31 as a BCL-2 and procaspase-8 interacting protein, and a caspase-8 substrate was, therefore, of great interest (Ng et al., 1997; Ng and Shore, 1998). The primary goal of my project was explore whether BAP31 could indeed regulate endogenous procaspase-8 during a physiological apoptotic pathway. This issue is addressed in Chapter 2, wherein a novel ER-localized procaspase-8L isoform is identified and characterized. The remainder of my thesis focuses on defining the role of BAP31 as a caspase-8 substrate. Chapter 3 demonstrates that stable expression of a caspase-resistant BAP31 mutant inhibits several features of Fas-induced apoptosis, highlighting the importance of BAP31 cleavage on this pathway. The mechanism by which BAP31 cleavage regulates caspase-8-initated pathways is further explored in chapter 4, where the ability of the p20 caspase cleavage fragment to induce Ca^{2+}-dependent proapoptotic cross-talk between the ER and mitochondria is documented. The relevance of this ER-mitochondria cross-talk during Fas- and caspase-8-mediated cyt.c release is addressed in Chapter 5. Finally, in the Chapter 6, I discuss possible mechanisms by which BAP31 may coordinate procaspase-8L activation and how BAP31 cleavage contributes to Ca^{2+} release from the ER during Fas-induced apoptosis.

Chapter 2

Procaspase-8L, a Novel Procaspase-8 Isoform that is Recruited to the BAP31 Complex at the Endoplasmic Reticulum

2.1 Rationale

The cytosolic tail of BAP31 shares sequence homology with the DEDs of initiator procaspase-8 and FADD, and can interact with procaspase-8 when both proteins are ectopically expressed in 293T cells (Ng et al., 1997; Ng and Shore, 1998). These observations suggest that BAP31 may bind and regulate endogenous procaspase-8 in response to certain death stimuli. In this chapter, I addressed this issue by exploring whether BAP31 interacted with endogenous procaspase-8 during E1A-oncogene induced apoptosis. E1A was chosen as a death inducer because we noted that procaspase-8 activation is an important event that occurs early on the E1A death pathway (Nguyen et al., 1998). Importantly, however, E1A induced activation of caspase-8 occurs independently of FADD-dependent death receptors (Nguyen et al., 1998), raising the possibility that other potential procaspase-8 regulatory complexes, such as BAP31, might achieve this role in this apoptotic pathway.

2.2 Abstract

BAP31 is an integral protein of the endoplasmic reticulum membrane and a substrate of caspase-8. Here, we describe a novel procaspase-8 isoform, procaspase-8L, which is ubiquitously expressed and selectively recruited to the BAP31 complex in response to apoptotic signaling by E1A. Procaspase-8L is characterized by the Nex domain, which extends procaspase-8/a at the N-terminus and is required for selective association of procaspase-8L with the BAP31 complex. Gene deletion identified BAP31 and related BAP29 as required for processing of procaspase-8L in response to E1A, by a FADD-independent mechanism that was blocked by BCL-2. Further, Bap29,31 deletion,

as well as a Nex-domain dominant-negative mutant, curtailed the activation of downstream caspases (IETDase and DEVDase) and cell death in response to E1A. Preferential recruitment of procaspase-8L by the BAP31 complex at the endoplasmic reticulum suggests an additional pathway for regulating initiator caspase-8 during apoptosis.

2.3 Introduction

Physiological cell death occurs in response to diverse signals, which initiate pathways that ultimately are coupled to complexes that activate the death machinery, typically comprised of caspases. To date, two caspase activation complexes have been identified: death-inducing signaling complexes (DISCs) of the tumor necrosis factor (TNF)-family of death receptors that activate initiator procaspase-8, via the adapter FADD, in response to extrinsic cytokine signals, and a mitochondrial-dependent complex, the apoptosome, that activates initiator procaspase-9 in response to multiple cell intrinsic death signals (Budihardjo et al., 1999; Earnshaw et al., 1999). After activation, caspase-8 and caspase-9 stimulate a downstream cascade of events by directly processing effector procaspases such as procaspase-3. The activated effector enzymes then cleave several hundred cellular proteins, causing apoptotic cell suicide (Earnshaw et al., 1999). An additional target of caspase-8, BID, is cleaved to tBID, which mediates cross-talk between death receptors at the plasma membrane and the apoptosome by inducing mitochondria to release a critical cofactor, cytochrome c (Korsmeyer et al., 2000).

BAP31 is a 28 kDa polytopic integral membrane protein that is ubiquitously expressed (Adachi et al., 1996; Kim et al., 1994; Mosser et al., 1994) and highly enriched

at the endoplasmic reticulum (ER) (Annaert et al., 1997; Ng et al., 1997) where it forms both a homo-oligomer and a hetero-oligomer with the closely related BAP29 (Adachi et al., 1996; Ng et al., 1997). A role for BAP31 as a potential regulator of apoptosis derived from its discovery as a predicted BCL-2/BCL-X_L associated protein (Ng et al., 1997). The 14 kDa cytosolic tail of human BAP31 contains a weak death effector and overlapping coiled-coil (DECC) domain, flanked on either side by identical caspase-8 recognition sites, and terminating at the C-terminus with a canonical KKXX ER retention signal (Ng et al., 1997; Ng and Shore, 1998). In addition to being a preferred caspase-8 substrate in vitro, the DECC domain of BAP31 can associate, albeit weakly, with procaspase-8 in over-expressing co-transfected 293T cells (Ng et al., 1997; Ng and Shore, 1998). Of note, two predominant protein isoforms of procaspase-8 have been identified to date, procaspase-8/a and procaspase-8/b, that differ only in a short sequence within the pro-domain that derives from the alternative splicing of *CASP8* exon 6 (Scaffidi et al., 1997). The two isoforms are typically expressed ubiquitously at equivalent levels, and appear to function interchangeably (Scaffidi et al., 1997).

Here, we describe a novel cellular isoform of procaspase-8, procaspase-8L, which is procaspase-8/a containing an N-terminal extension (Nex domain) of 59 amino acids. This domain allows selective recruitment of procaspase-8L to the BAP31 complex in response to apoptotic signaling by the model oncogene, adenovirus E1A. Gene deletion indicates that the BAP proteins are required for E1A-induced processing of procaspase-8L and contribute to downstream activation of caspases and cell death.

2.4 Materials and Methods

Antibodies and Reagents- Calnexin monoclonal antibody was purchased from Signal Transduction Labs, anti-FLAG M2 monoclonal antibody and M2 FLAG gel were from Sigma, and anti-HA 12CA5 monoclonal antibody from Babco. Rabbit antibody against the p18 catalytic subunit of human caspase-8 was from S. Roy and D. Nicholson, anti-Apo-1 was from P. Krammer, and anti-procaspase-8 N2 monoclonal antibody (raised against the caspase-8 prodomain) from M. Peter. Rabbit anti-mouse caspase-8 (p18) was from Santa Cruz Biotechnologies. Rabbit anti-BAP31 and anti-Bap29 antibodies have been described (Adachi et al., 1996; Ng et al., 1997). The procaspase-8L Nex domain peptide specific antibody was generated at Research Genetics. Rabbits were immunized with the peptide NH_2-EHVELGRLGDSETA-COOH, conjugated at its amino terminus to KLH, and serum was affinity purified against the immunizing peptide. A second anti-Nex antibody generated in our lab against a recombinant GST-Nex domain fusion protein and absorbed on a GST column, was used in Figure 2.2D.

Cell culture, virus infection and apoptosis assays - Human KB epithelial and H1299 lung carcinoma cells were maintained as described previously (Ng et al., 1997). H9 lymphocytes were cultured in RPMI 1640 including 10mM HEPES and supplemented as above. KB cells stably expressing HA-BCL-2, GFP-Flag, BAP31-Flag or crBAP31-Flag have been described (Nguyen et al., 1998; Nguyen et al., 2000) and were grown as above in the presence of 350 mg/ml geniticin (GibcoBRL). KB cells stably expressing both crBAP31-Flag and HA-BCL-2 were constructed by transfecting HA-BCL-2 stable transfectants with crBAP31-Flag in pcDNA3.1/Hygro(-) and selecting for clones resistant to both geneticin and hygromycin. Wild type and *Bap29,31*-null ES cells were grown at 37°C and 7.5% CO_2 on embryonic fibroblast feeders in KNOCKOUT D-MEM™

(GibcoBRL) supplemented with 100u/mL streptomycin sulfate and penicillin, 15% fetal bovine serum, 2mM L-glutamine, non-essential amino acids, 1mM β-mercaptoethanol, in the presence of leukemia inhibitory factor (LIF). ES cells were differentiated into embryoid epithelial and fibroblast-like cells as follows; cells were propagated on gelatin coated dishes for 3 passes (8 days) in the absence of LIF to dilute out feeder cells, and then replated on non-gelatinized dishes allowing monolayers of differentiated cells to form. Infection of cells with Ad5 *dl*520E1B$^-$, which expresses the 243 residue form (12 S) of E1A and no E1B products, was performed as described (Ng et al., 1997) and ≥98% of cells were found to be expressing E1A 20 h post-infection. IETD-amc and DEVD-amc caspase activity assays (Upstate Biotechnology) and Annexin V staining (BioVision) were conducted according to the manufacture's protocols

RACE Analysis and Expression Constructs - PolyA$^+$ mRNA from KB cells was isolated using the Oligotex Direct mRNA Kit (Quiagen). Both 5' and 3' RACE were performed using the SMART™ RACE cDNA Amplification Kit (Clonetech). Primers 5'-CGGGATCCGATTCTGCCTTTCTGCTGG and 5'-GCCAAGCTTTCAATCAGAA GGGAAGAC-3' were used to PCR amplify the procaspase-8L reading frame. All PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen), and at least three independent clones of each product were sequenced on both strands. Procaspase-8 and procaspase-8L, each tagged at their carboxy terminus with HA, were created using standard PCR techniques and cloned into pcDNA3. For the Nex-EGFP construct, standard PCR techniques were used to create the Nex domain with a C-terminal Flag tag; this product was then cloned into the pEGFP-N3 Vector so that it was upstream and

inframe with EGFP. Construction of pcDNA3 vectors expressing crBAP31-Flag and BAP31-Flag has been described (Nguyen et al., 2000). pcDNA3 Vector expressing Flag-tagged PAIP-I or 12 S E1A were gifts from N. Sonenberg and R. Marcellus, respectively.

Immunoprecipitation - For immunoprecipitation of the BAP31 complex, 1-2×10^7 cells per sample were washed once in phosphate-buffered saline (PBS) and lysed for 30 min at 4°C in 2 ml lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1μg/ml aprotinin, 1μg/ml leupeptin, and 1μg/ml pepstatin). The cell lysate was centrifuged at 12000×g for 15 min to remove insoluble membrane and debris, and subsequently precleared with protein G Sepharose (Pharmacia) for 1 hr. crBAP31-Flag or BAP31-Flag was then precipitated for 4 h at 4°C with FLAG M2 Gel, and washed 5 times with lysis buffer. The BAP31 complex was eluted from the beads by Flag peptide competition or by boiling in SDS sample buffer. DISC analysis was done exactly as described (Medema et al., 1997). All samples were analyzed by SDS-PAGE and Western blotting.

Subcellular Fractionation - KB cells were fractionated as described by Goping et al. (Goping et al., 1998) with minor modifications.

Transient Transfection - For Figure 2.4B, 3×10^5 H1299 cells were transfected with Lipofectamine Plus (GibcoBRL) and 1 μg of pcDNA3 vector, or this vector expressing either procaspase-8/a-HA or procaspase-8L-HA, together with 0.1 μg of vector expressing luciferase. 3 hr post transfection, the cells were incubated in medium

containing or lacking 50 μM zVAD-fmk for 24 h and adherent and floating cells were then collected, washed, lysed and total luciferase activity was measured as described (Ng et al., 1997). In Figure 4A, 7×10^6 H1299 cells were transfected as described above with 5 μg of each of the indicated constructs in the presence of zVAD-fmk. After 24 hr, the cells were lysed and BAP31-Flag and PAIP-1-Flag were immunoprecipitated as described above. In Figure 6, 2×10^5 KB cells in 24 well clusters were transfected with the indicated plasmids using Lipofectamine 2000 (GibcoBRL).

Targeted Disruption of Bap29 and Bap31 - To target the *X*-linked *Bap31* gene, a targeting construct comprising 1.2 kb of intron 1, exon 2 (containing the start codon) fused in-frame with the full length Bap31 coding sequence, an internal ribosome entry site (IRES), a promotorless neomycin phosphotransferase coding sequence, a polyadenylation signal, and 4.7 kb of intron 3 was created. This targeting construct contained three *lox*P sites; the first in the intron 1-derived region, the second between the Bap31 coding sequence and the IRES, and the third between the neomycin cassette and the polyadenylation signal. C57BL/6 ES cells were electroporated with the linearized targeting vector and G418-resistant colonies were screened for homologous recombinants by PCR and Southern blotting. Bap31 protein expression in the gene-targeted cells was indistinguishable from parental cells. A complete disruption of the *Bap31* gene was achieved by transient expression of Cre recombinase. Clones with only a Cre-mediated deletion of the IRES and neo cassette were isolated and targeted with a linerized targeting construct composed of a 250 bp homology region, a neomycin phosphotransferase coding sequence fused in-frame to the *Bap29* translation initiation codon, a polyadenylation signal and a 3.5 kb

homology region. In this case, homologous recombination resulted in loss of *Bap29* expression from the targeted allele. To generate cells negative for both *Bap29* and *Bap31*, *Bap29*-/- clones underwent another round of transient Cre expression.

2.5 Results and Discussion

2.5.1 Identification of Procaspase-8L

The adenovirus type 5 vector, Ad5 *dl*520E1B$^-$ (Shepherd et al., 1993) encodes 12 S E1A mRNA and no E1B products, generating the 243 amino acid E1A oncoprotein as the only vector expression product. Infection of human KB epithelial cells with this Ad E1A vector stimulates activation of caspases including caspases –8 and -3, cleavage of the caspase-8 preferred substrate BAP31 and the caspase-3 preferred substrate, PARP, and apoptosis (Boulakia et al., 1996; Ng et al., 1997; Nguyen et al., 1998). To observe the recruitment of procaspase-8 to the caspase-sensitive BAP31 complex during E1A-induced apoptosis, therefore, a KB cell line was created that stably expresses an epitope tagged caspase-resistant mutant, crBAP31-Flag (Figure 2.1A), in which the mutant was expressed at levels only slightly higher than that of endogenous BAP31 (Nguyen et al., 2000). As expected, crBAP31, but not endogenous BAP31 or wt BAP31-Flag, remained structurally intact during E1A-induced apoptosis (Figure 2.1B).

In Figure 2.1C, crBAP31-Flag cells, with or without stable co-expression of BCL-2, were mock-infected or infected with Ad E1A for 40 h, and cell lysates subjected to precipitation with anti-Flag antibody and analysis by Western (immuno) blotting with antibodies either against the p18 catalytic subunit of caspase-8 or against BAP31. An approximately 62 kDa product (labeled procaspase-8L) that reacted with antibody against

p18 was observed in crBAP31-Flag precipitates in the presence (bottom panel, lane 4) but not absence (lane 3) of E1A signaling. In contrast, the conventional 55 and 53 kDa procaspase-8/a and procaspase-8/b isoforms observed in cell lysates (Figure 2-1C top panel) were not detected in the crBAP31-Flag precipitates. Cells expressing a control protein, GFP-Flag, did not yield any anti-p18 reactive product in the anti-Flag precipitate (Figure2.1C, bottom panel). In KB cells stably expressing both crBAP31-Flag and anti-apoptotic BCL-2, processing of the conventional forms of procaspase-8/a and –8/b in response to E1A was inhibited (Fig. 2.1C, lanes 5 and 6 top panel), endogenous BAP31 was not cleaved (as judged by the lack of appearance of the p20 product derived from caspase cleavage of endogenous BAP31; middle panel), and apoptosis was blocked (Nguyen et al., 1998). BCL-2, however, did not interfere with the E1A-induced recruitment of the 62 kDa product to the crBAP31 complex; the apparent increase in levels of this product that associated with crBAP31 in the presence of BCL-2 (compare lane 4 lane 6, bottom panel) may arise because BCL-2 blocks its processing (see below) and, therefore, preserves the pool of intact precursor protein. In addition, procaspase-8L recruitment was not blocked by the pan-caspase inhibitor zVAD-fmk (Fig. 2.1C, bottom panel lanes 7 and 8), although caspase activation and cell death were inhibited (data not shown). As expected, the p20 caspase cleavage product of endogenous BAP31 that was generated in the absence of BCL-2 (12) co-precipitated with crBAP31-Flag (middle panel, lane 4) because it forms a stable oligomer with intact BAP31 (Ng and Shore, 1998). Compared to procaspase-8/a and –8/b, the 62 kDa product was not clearly detectable in whole cell lysates with anti-p18 antibody, suggesting that it might be a minor product.

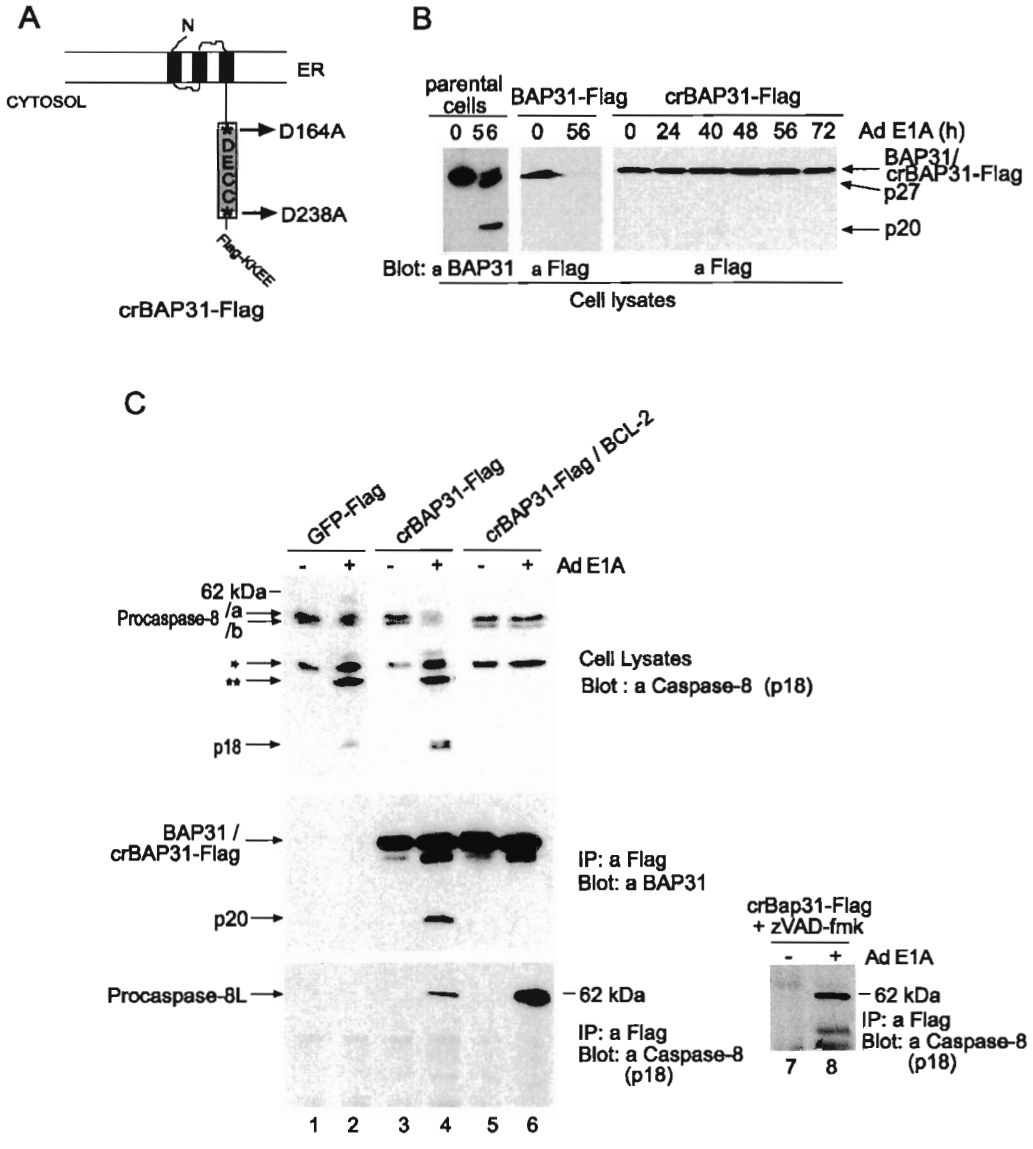

Figure 2.1. Procaspase-8L is recruited to the BAP31 complex during E1A-induced apoptosis. (A) Schematic of crBAP31-Flag in the ER. Asp164 and Asp238 were mutated to Ala and a Flag epiptope was inserted directly in front of the KKEE ER retrieval sequence. (*) caspase recognition Asp residues. (B) crBAP31-Flag is not cleaved during E1A-induced apoptosis. Parental KB cells or KB cells stably expressing wtBAP31-Flag or crBAP31-Flag were treated with adenovirus type 5 (Ad5) *dl*520E1B$^-$ for the indicated times and endogenous BAP31, BAP31-Flag or crBAP31-Flag cleavage was analyzed by SDS-PAGE and immuno-blotting with anti-BAP31 or anti-Flag antibodies. (C) A novel approx. 62 kDa form of procaspase-8, designated procaspase-8L, is recruited to the BAP31 complex in response to E1A signaling. KB cells stably expressing GFP-Flag, crBAP31-Flag, or crBAP31-Flag and BCL-2 were mock infected or infected (E1A) with Ad5 *dl*520E1B$^-$ for 40 hr and immunoprecipitation (IP) was conducted with anti-Flag M2 gel followed by SDS-PAGE and Western blotting with anti-BAP31 (middle panel) or anti-caspase-8 antibody specific for the p18 subunit (bottom panel). Where indicated, cells were cultured in the presence of zVAD-fmk (50 μm). Immuno blots of the corresponding total cell lysate (5% of the input used for immunoprecipitation) are also shown (top panel). The positions of the conventional procaspase-8 isoforms, designated /a and /b, their processing intermediates (asterisks), the caspase-8 catalytic subunit (p18), procaspase-8L, crBAP31-Flag, endogenous BAP31 and its p20 cleavage product, are shown.

2.5.2 Molecular Characterization of Procaspase-8L

An extensive analysis of the 5' ends of procaspase-8 mRNA transcripts in KB epithelial cells was conducted using 5' Rapid Amplification of cDNA Ends (RACE). Five transcripts were identified that differ in sequence upstream of *CASP8* exon 3 (GenBank accession nos. AF422925 – 22929). Exon 3 contains the ATG start site of translation for conventional procaspase-8/a and –8/b (Grenet et al., 1999) (see Fig. 2.2A). Four of the transcripts contained inframe stop codons upstream of exon 3 whereas one transcript, accession no. AF422925, contained an upstream ATG within a strong Kozak translational initiation consensus sequence (GCC*ATG*G), which is inframe with the procaspase-8 open reading frame (ORF) initiating within exon 3 (Fig. 2.2A). This was the most 5' ATG codon in the derived transcript, which together with the surrounding consensus sequence, suggested that it might function as the preferential site of translation initiation in this transcript (Kozak, 1989). If so, the predicted N-terminal extended ORF encodes 59 amino acids upstream of the conventional procaspase-8 translation initiation site, and was designated the N-terminal extended (Nex) domain. The Nex domain was continuous with the entire procaspase-8/a ORF as determined by PCR and sequencing of first strand KB cell cDNA, using primers specific for the 5'end of the Nex domain and the 3' end of the caspase-8 p10 subunit (Fig. 2.2B). Importantly, the Nex domain could not be connected to any transcript other than procaspase-8/a by 3' RACE. The extended ORF consisting of the Nex domain linked to the procaspase-8/a ORF codes for a protein with a predicted molecular size of 61.9 kDa, which is consistent with the size of the 62 kDa anti-p18 reactive protein found associated with the BAP31 complex, and was designated procaspase-8L (long).

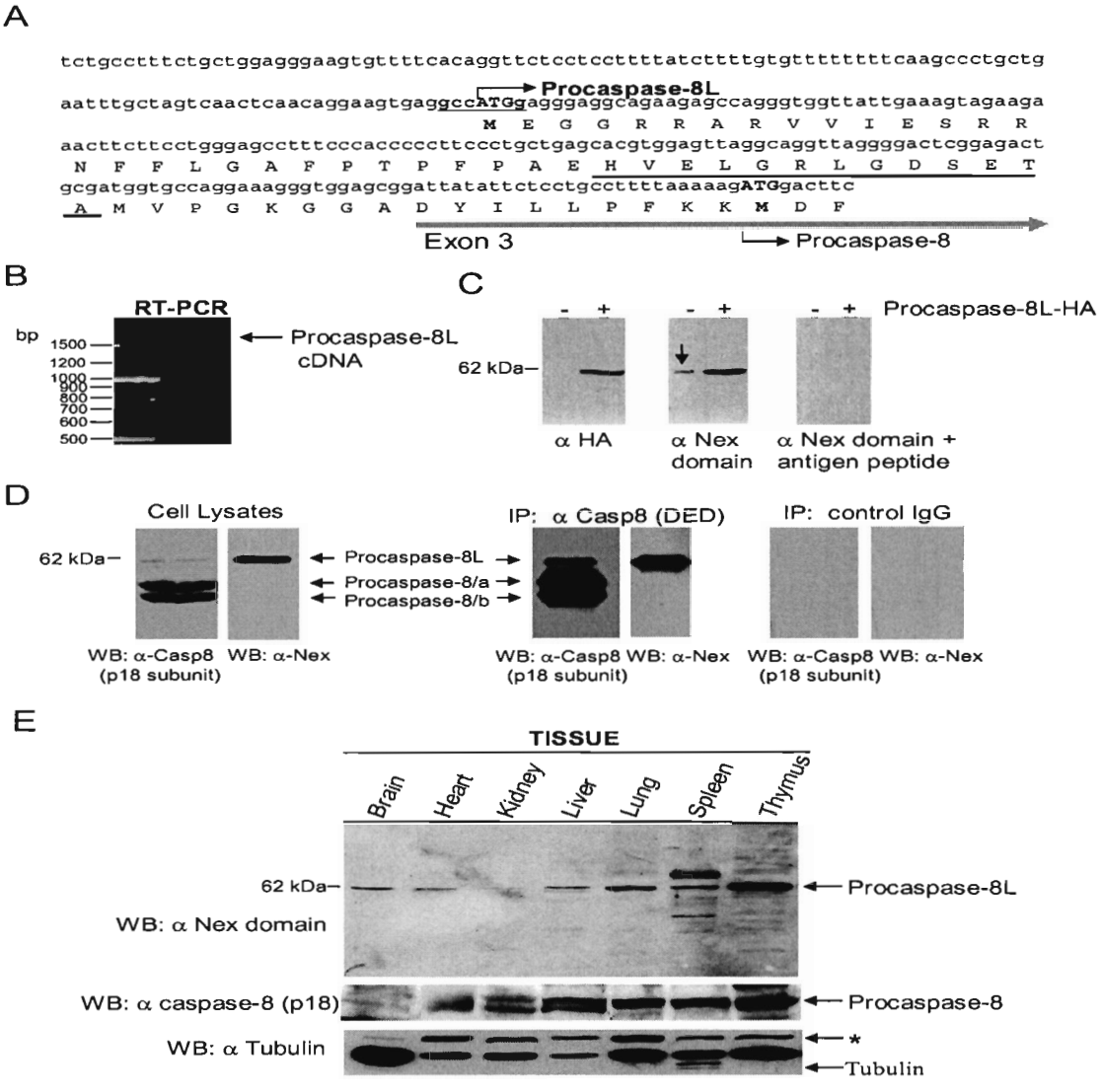

Figure 2.2. Characterization and cloning of procaspase-8L. (A) Deduced amino acid sequence of the predicted N-terminal extension (Nex) domain of procaspase-8L. The predicted procaspase-8L upstream start codon and Kozak translation initiation consensus sequence are shown in bold. The positions of exon 3 and the canonical procaspase-8/a and –8/b start site are indicated. The sequence of the peptide used for the procaspase-8L Nex domain antibody production is underlined in black. (B) PCR of first strand cDNA derived from KB cell mRNA using primers specific for the 5'end of the Nex domain and the 3' end of the canonical caspase-8 p10 subunit demonstrate that the Nex domain of procaspase-8L is connected to the entire procaspase-8/a open reading frame (ORF). The RT-PCR product is indicated (arrow). (C) Affinity purified rabbit polyclonal antibodies raised to the Nex domain of procaspase-8L detect an endogenous protein of approximately 62 kDa in human H1299 lung carcinoma cells (arrow, middle panel) and ectopic expressed procaspase-8L-HA. Cells were transfected with vector (-) or with vector expressing HA-tagged procaspase-8L (+) in the presence of 50 μM zVAD-fmk and, after 24 hr, total cell lysates were analyzed by Western blotting with the indicated antibodies, in the presence or absence of 10 μM immunizing peptide. (D) Cell lysates were subjected to immunoprecipitation with an anti-caspase-8 monoclonal antibody recognizing the prodomain (Scaffidi et al., 1997). Lysates and precipitates were analyzed by blotting with anti-caspase-8 p18 subunit specific antibody or anti-Nex domain antibody. (E) Tissue distribution of procaspase-8L. The indicated murine tissue lysates were analyzed by immuno blotting as in (D). The blot was reprobed with anti-tubulin monoclonal antibody for loading control (*, cross reacting product).

Antibody raised against a peptide sequence within the Nex domain (solid black underline in Fig. 2.2A) detected an endogenous 62 kDa protein in H1299 cells (arrow in Fig. 2.2E, middle panel), and demonstrated enhanced reactivity toward procaspase-8L protein obtained by over-expression in these cells of hemagglutinin (HA) -tagged procaspase-8L cDNA by transient transfection (Fig. 2.2C). The specificity of the anti-Nex domain antibody in Western blot was demonstrated by competitive inhibition with the Nex domain antigen peptide (Fig. 2.2C, right panel). Moreover, recovery of procaspase-8 isoforms from cell lysate with a precipitating procaspase-8 antibody (Scaffidi et al., 1997) and blotting of the precipitate with anti-Nex detected the 62 kDa product (Fig. 2.2D). As predicted, probing the precipitate with antibody against the common p18 subunit revealed that procaspase-8L is expressed at a considerably lower level than the two main isoforms (Fig. 2.2D). Analysis of murine tissues by immunoblot suggested that procaspase-8L is widely expressed (Fig. 2.2E). Taken together, these results suggest that procaspase-8L is a novel procaspase-8 isoform that is expressed at the protein level and contains a unique N-terminal Nex domain.

2.5.3 Subcellular Distribution of Procaspase-8L

KB cells were fractionated into P1 nuclear, heavy membrane (HM), light membrane (LM), and cytosolic S-100 fractions and the presence of procaspase-8L in each fraction was examined with the anti-Nex domain antibody. As shown in Figure 2.3A, procaspase-8L distributed between the S100 cytosolic fraction and the LM fraction enriched in microsomes, as indicated by the ER marker, calnexin. In contrast, procaspase-8/a and -8/b were located exclusively in the S100 fraction. Further investigation using analytical fractionation of rodent liver membranes revealed that

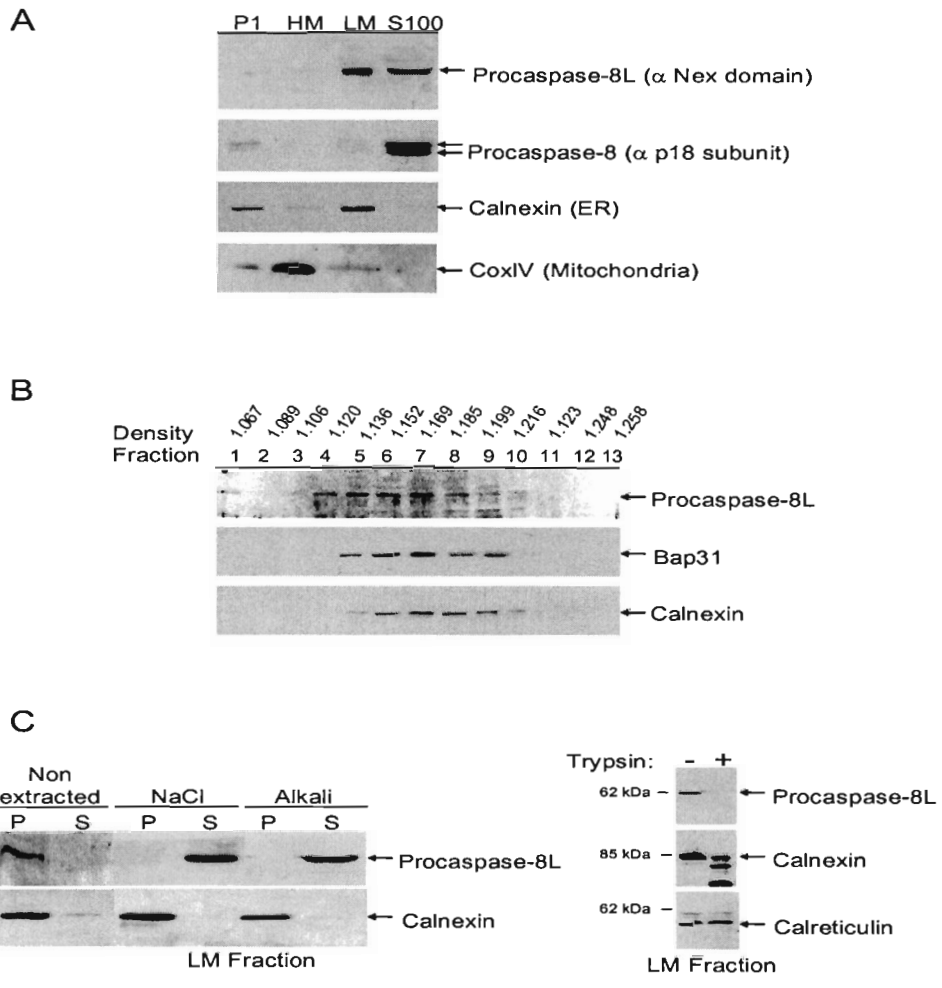

Figure 2.3. Subcellular distribution of procaspase-8L. (A) KB cells were homogenized in isotonic buffer and the P1 nuclear (500×g pellet), heavy membrane (HM) (9000×g pellet), light membrane (LM) (100 000×g pellet) and cytosolic S-100 fractions were separated by differential centrifugation. The fractions were analyzed for the presence of procaspase-8L, procaspase-8, the ER marker, calnexin, and the mitochondrial marker, cytochrome c oxidase subunit IV (CoxIV) by immuno blotting with the respective antibodies. (B) Sucrose density gradient fractions of rodent liver membranes were analyzed for the presence of procaspase-8L, BAP31 and calnexin as in (A). (C) Procaspase-8L is peripherally associated with the cytosolic face of the light membranes. (Left) The LM fraction from KB cells was subjected to extraction with 0.5M NaCl or alkali (0.1M $NaCO_3$, pH 11.5) and, after centrifugation, the membrane pellets (P) (lanes 1, 3) and supernatants (lanes 2, 4) were analyzed as in (A). (Right) The LM fraction was incubated with (+) or without (-) trypsin and the integrity of procaspase-8L, calnexin (ER transmembrane protein), and BiP (ER lumenal protein) assessed by immuno blotting. TritonX-100 was added to 1% in one reaction to demonstrate that BiP was trypsin sensitive following solubilization of the LM microsomal fraction.

procaspase-8L and Bap31 co-sedimented with near identical median densities that were slightly lower than the median density of calnexin (Fig. 2.3B). In contrast to an integral membrane protein like calnexin, procaspase-8L was extracted from the LM fraction with high salt concentrations or alkali (pH11.5) (Fig. 2.3C , left panel), suggesting that it is peripherally associated with microsomal membrane. Furthermore, treatment of the LM fraction with trypsin showed that procaspase-8L was sensitive to digestion, whereas an ER lumenal protein, BiP, was completely resistant (Fig. 2.3C, right panel). Therefore, the initial location of procaspase-8L at the cytosolic face ER may provide a nearby pool for recruitment to the BAP31 complex following E1A stimulation.

2.5.4 Procaspase-8L Recruitment to the BAP31 Complex and Cleavage in Response to E1A Signaling

Immunoblot analysis of KB cells with anti-Nex domain antibody detected endogenous procaspase-8L, which was cleaved to an approx. 35 kDa protein in response to E1A expression (Fig. 2.4A). Similar results were observed in cells expressing crBAP31-Flag (not shown) and, in both cases, this apparent procaspase-8L processing was blocked by BCL-2 or by the wide spectrum caspase inhibitor, zVAD-fmk (Fig. 2.4A and 2.4B; data not shown). Given that the size of the cleavage product detected with the anti-Nex domain antibody is similar to the predicted size of the procaspase-8L prodomain (33 kDa), the observed proteolysis likely separates the prodomain from the p20 and p10 catalytic subunits.

Procaspase-8L was maximally recovered in crBAP31-Flag immuno-precipitates 40 h after introducing E1A into cells, as determined by immunoblot analysis with anti-Nex (Fig. 2.4C, top left panel), and this corresponded to the time when both procaspase-

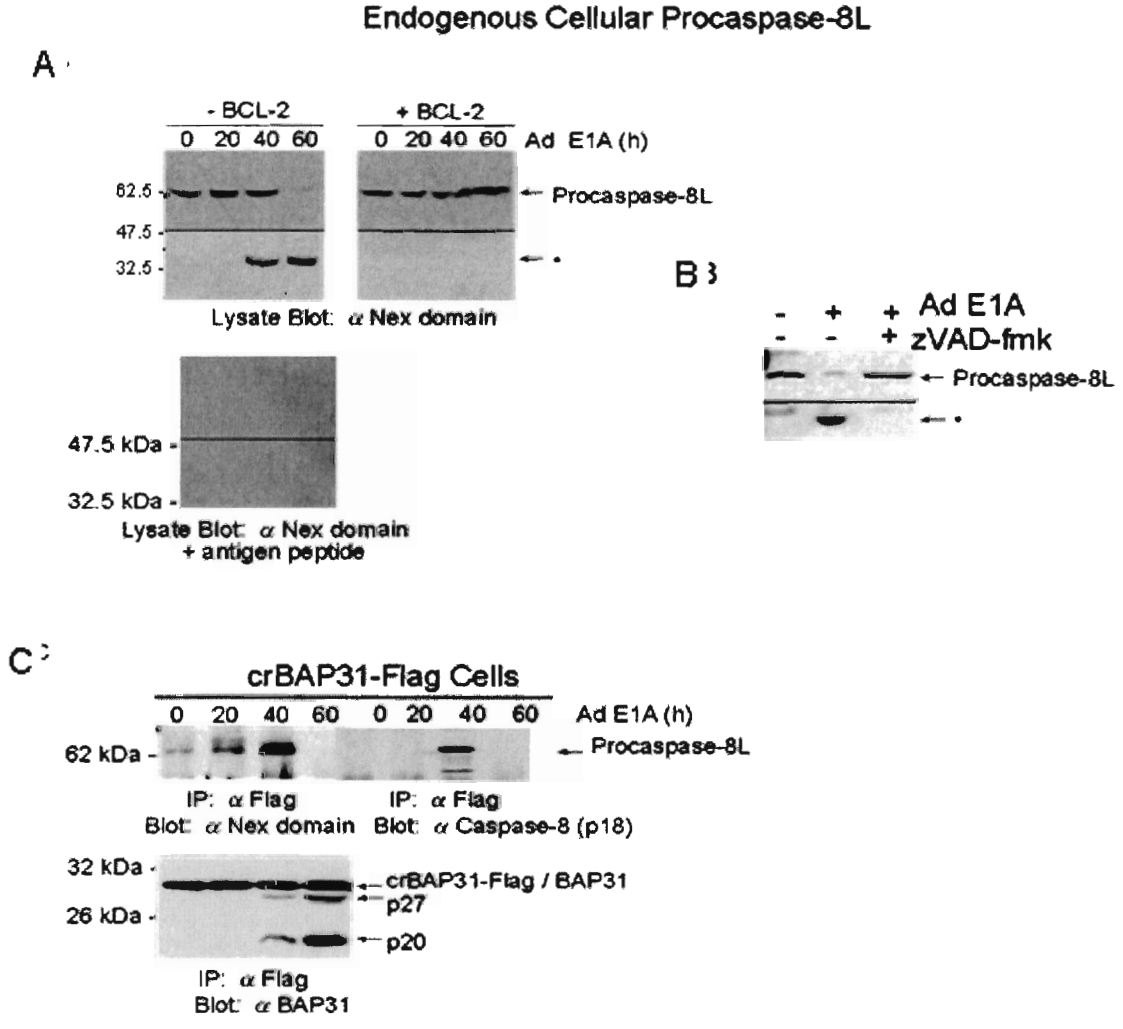

Figure 2.4. Endogenous procaspase-8L is recruited to the BAP31 complex and cleaved during E1A-induced apoptosis. (A) Cleavage of procaspase-8L is blocked by BCL-2. Parental KB cells (- BCL-2) or KB cells stably expressing BCL-2 (+ BCL-2) were infected with Ad5 *dl*520E1B⁻ for the indicated times and Western blots were developed with anti-Nex antibody, in the absence (top panels) or presence (bottom panel) of the Nex immunizing peptide (10μM). (B) Cleavage of procaspase-8L is inhibited by z-VAD-fmk. Cells were treated as in (A) in the absence or presence of 50 μM zVAD-fmk. (C) Recruitment of procaspase-8L to the BAP31 complex. KB cells stably expressing crBAP31-Flag were treated as in (A) and the BAP31 complex was immunoprecipitated (α Flag IP) at the indicated times post-infection. Immuno blots of the precipitates were developed with anti-Nex antibody (upper left panel), then stripped and reprobed with anti-caspase-8 (p18) antibody (upper right panel) or anti-BAP31 antibody (lower panel). The anti-Nex domain and anti-caspase-8 (p18) antibodies detected an identical 62 kDa protein. The p20 and p27 cleavage products of endogenous BAP31 (that heterodimerize with crBAP31-Flag) are indicated. A representative experiment is shown.

8L processing (Fig. 2.4A) and caspase cleavage of endogenous BAP31 (Fig.2.4C, bottom panel) had been initiated. The anti-Nex blot of the crBAP31-Flag precipitates was then stripped and re-probed with antibody against the p18 subunit of caspase-8. Again, a product was detected that exactly co-migrated with the anti-Nex reactive product, showing maximal accumulation with crBAP31-Flag at 40h post-infection with Ad E1A (Fig. 2.4C, top right panel). By 60 h, procaspase-8L cleavage was completed (Fig. 2.4A) and anti-Nex and anti-p18 reactive product was no longer detected in association with crBAP31-Flag (Fig. 2.4C). Therefore, the association observed between procaspase-8L and crBAP31-Flag coincides with the proteolytic maturation of procaspase-8L.

2.5.6 Selective Recruitment of Procaspase-8L to the BAP31 Complex

Consistent with the observation that endogenous procaspase-8/a and –8/b were not detectably recruited to crBAP31-Flag following E1A expression (Fig. 2.1E), over-expression of procaspase-8/a-HA and procaspase-8L-HA by transient co-transfection of the respective cDNAs with cDNA encoding BAP31-Flag in H1299 cells showed a marked preference for interactions between the BAP31 complex and procaspase-8L (Fig. 2.5A, lanes 4 and 5). Neither procaspase exhibited a detectable association with the control protein, PABP Interacting Protein-I-Flag (PAIP-I-Flag). Transfections were conducted in the presence of zVAD-fmk. In the absence of the caspase inhibitor, ectopically expressed procaspase-8/a and procaspase-8L underwent processing (data not shown) and potently induced cell death, as assessed by co-transfection with a luciferase reporter and measurement of luciferase activity (Fig. 2.5B) or by co-transfection with a GFP reporter and measurement of the apoptotic morphology of GFP-transfected cells (not shown).

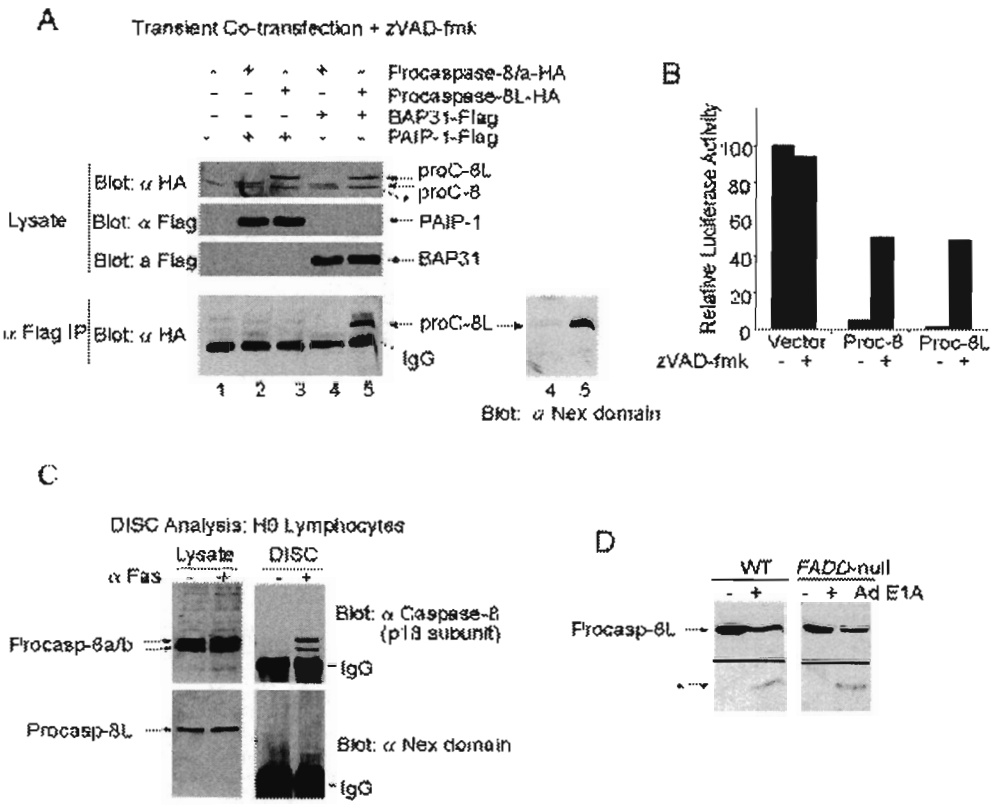

Figure 2.5. Procaspase-8L selectively associates with the BAP31 complex. (A) Anti-Flag immunoprecipitates from H1299 cells cotransfected with the indicated expression constructs in the presence of 50 μM zVAD-fmk were analyzed by SDS-PAGE and Western blotting with anti-HA or anti-Nex domain antibodies. Note that only procaspase-8L-HA immunoprecipitated with BAP31-Flag. (*) represents a non-specific protein detected by anti-HA antibody in lysates. (B) Procaspase-8L induces loss of cell viability when ectopically expressed. H1299 cells were transfected with a luciferase expression construct and pcDNA3 vector expressing either procaspase-8/a-HA or procaspase-8L-HA, in the absence or presence of 50 μM zVAD-fmk and luciferase activity was measured 24 h later. (C) Procaspase-8L is not detected in the Fas DISC. The DISC was immunoprecipitated following stimulation of H9 lymphocytes with anti-Apo-1/Fas and the presence of procaspase-8L and procaspase-8 in the precipitates was assessed by immuno blotting as in Fig. 2.4C. IgG, immunoglobulin heavy chain. (D) Wt or *Fadd*-deficient mouse embryo fibroblasts (Yeh et al., 1998) were infected with Ad5 *dl*520E1B$^-$ for 50 h and procaspase-8L cleavage was analyzed as in Figure 2.4A.

Procaspase-8/a and –8/b are recruited to DISCs of the TNF receptor family upon receptor stimulation (Scaffidi et al., 1997). To determine whether procaspase-8L is also a component of this signaling complex, we stimulated H9 lymphocytes with the agonistic Fas antibody, anti-Apo-1 (Kischkel et al., 2001), and immunoprecipitated the DISC. As

expected, procaspase-8/a and –8/b were both recruited to the Fas DISC in a stimulation dependent manner. In the same DISC precipitation, however, no procaspase-8L was detected with the anti-Nex antibody (Fig. 2.5C), suggesting that if procaspase-8L associates with this complex it is a minor component of it. Moreover, procaspase-8L cleavage was not impaired in *Fadd*-null primary mouse embryo fibroblasts (Yeh et al., 1998) in response to E1A (Fig. 2.5D) nor do these same cells exhibit detectable resistance to Ad E1A-induced cell death (Nguyen et al., 1998; Yeh et al., 1998), suggesting that E1A-induced processing of procaspase-8L is FADD-independent.

2.5.7 Nex Domain Dominate-Negative Mutant Inhibits E1A-induced Apoptosis

Since the procaspase-8L Nex domain is required for recruitment of the proenzyme to the BAP31 complex in response to E1A, expression of the Nex domain on its own might be expected to exert a dominant-negative influence on E1A death signaling. In Figure 2.6, KB cells were transiently co-transfected with a plasmid encoding 12S E1A and plasmid encoding either Nex-EGFP or EGFP. Compared to EGFP, Nex-EGFP significantly inhibited both E1A-induced cleavage of the caspase-8 preferred substrate, IETD-amc (Fig. 2.6A), and E1A-induced appearance of Annexin V positive cells (Fig. 2-6C). Of note, expression of a catalytically inactive full length procaspase-8L DN, in which the catalytic cys was mutated to ala, also inhibited E1A induced apoptosis to a similar extent as Nex-EGFP (data not shown). Nex-EGFP was expressed at a similar level as EGFP and did not affect the expression of E1A (Fig. 2.6B).

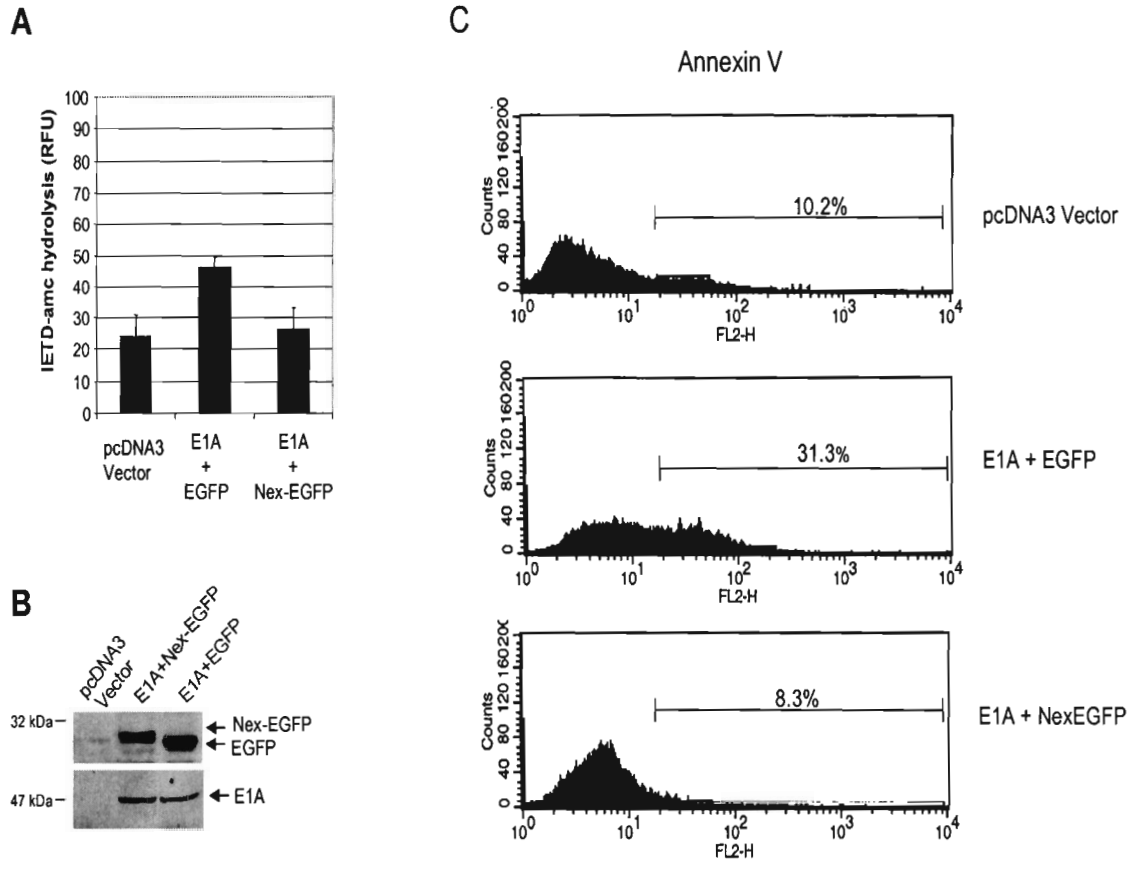

Figure 2.6. E1A-induced caspase-8 activity and apoptosis is inhibited by a procaspase-8L DN mutant. (A) KB cells were transiently co-transfected with plasmids encoding 12S E1A and Nex-Flag-EGFP or 12S E1A and Flag-EGFP and 36h post transfection equivalent amounts of cell lysate were tested for their ability to hydrolyze the caspase-8 preferred substrate IETD-amc. Shown is the mean of four independent experiments. (B) Cell lysates from (A) were analyzed by SDS-PAGE and immunoblotting with antibodies against E1A or Flag. (C) As in (A) except cells were collected, stained with Annexin V and analyzed by flow cytometry. Transfection efficiency was estimated to be 20-30% by analyzing EGFP positive cells by immunofluorescence (not shown).

2.5.8 E1A-induced Cleavage of Procaspase-8L is Inhibited in Double *Bap29*- and *Bap31*-null Cells

BAP31 is part of large complex that includes both BAP31 homo-oligomers and hetero-oligomers comprising the closely related BAP29 (Adachi et al., 1996). To

examine the contribution of these proteins to procaspase-8L processing in response to E1A, mouse embryonic stem (ES) cells deficient in *Bap31* and *Bap29* were generated by gene targeting (S. Kuppig and M. Reth, unpublished; see Materials and Methods) (Fig. 2.7A), and both wild type and gene-deleted cells were subjected to growth conditions that favor differentiation into epithelial- and fibroblast-like cells. The levels of procaspase-8L were similar in wild type and *Bap29,31*-null cells and E1A protein was expressed at equal levels in all cell types following infection with Ad E1A (Fig. 2.7B and data not shown). Following expression of E1A in wild type cells, procaspase-8L was cleaved, generating the N-terminal fragment detected by anti-Nex domain antibody (Fig. 2.7B). In *Bap29,31*-null cells, on the other hand, this processing was strongly impaired (Fig. 2.7B) and the double-null cells exhibited a significant decrease in their ability to hydrolyze the caspase-8 preferred substrate, IETD-amc, and the caspase-3 preferred substrate, DEVD-amc, in response to E1A (Fig. 2.7D and 2.7E). Procaspase-8L processing was not significantly altered in either *Bap31* or *Bap29* single knockout cells (data not shown), suggesting that Bap31 and Bap29 can functionally complement one another. Interestingly, procaspase-8/a and -8/b cleavage was also reduced in the *Bap29,31*-null cells (Fig. 2.7C), consistent with idea that activated procaspase-8L might directly cleave conventional procaspase-8/a and -8/b. Importantly, E1A-induced cell death in *Bap29,31*-null cells was reproducibly less than in either wt or *Bap31*-null cells (Fig. 2.7F).

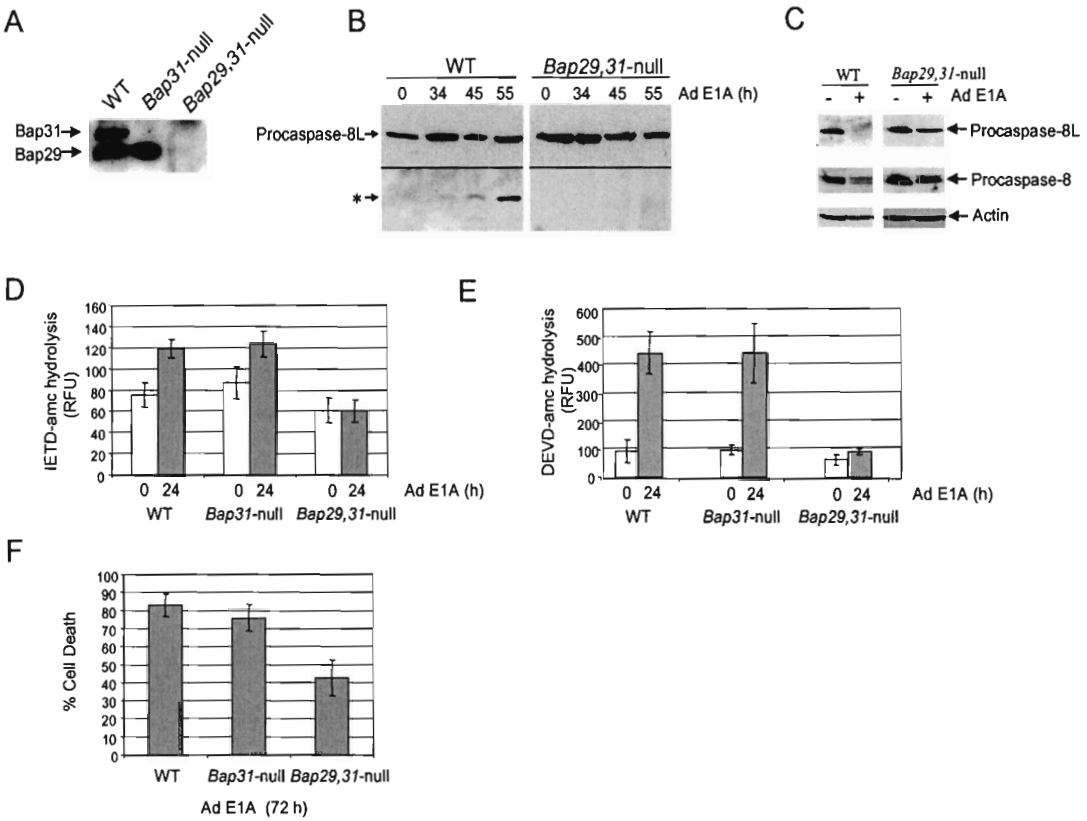

Figure 2.7. Procaspase-8L processing is inhibited in *Bap29,31*-null cells. (A) Loss of *Bap29* and *Bap31* expression was confirmed by immunoblotting with anti-BAP31 and anti-Bap29 antibodies. (B) E1A-induced procaspase-8L cleavage is inhibited in *Bap29,31*-null ES cells. ES cells were differentiated into epithelial- and fibroblast-like cells as described in Materials and Methods, and infected with Ad5 *dl*520E1B$^-$ for the indicated times. Procaspase-8L cleavage was analyzed as in Figure 3A. (C) As in (B), procaspase-8L and procaspase-8/a and -8/b cleavage was analyzed with anti-Nex and anti-caspase-8 (p18) antibodies. (D) Decreased caspase-8 activity in *Bap29,31*-null cells. Aliquots of lysate from wt and *Bap29,31*-null cells (containing equivalent protein concentrations) stimulated with E1A for 24h (time of maximum caspase activity) were tested for their ability to hydrolyze the preferred caspase-8 substrate IETD-amc. Shown is the average of three independent experiments. RFU, relative fluorescence units. (E) Decreased DEVD-ase activity in *Bap29,31*-null cells. As in (C) except lysates were tested for their ability to hydrolyze the caspase-3 preferred substrate DEVD-amc. (F) Cell death was measured by trypan blue exclusion 72 hrs post infection. Shown is a representative of 4 independent experiments.

Collectively, these results identify the novel isoform procaspase-8L whose Nex domain allows preferential recruitment of procaspase-8L to the BAP complex in the ER in response to apoptotic signaling by oncogenic E1A. Although E1A likely triggers

several pro-apoptotic pathways (Breckenridge and Shore, 2000; Lowe, 1999), gene deletion identified the BAP proteins as directly contributing to procaspase-8L processing, activation of downstream caspases, and cell death. The involvement of procaspase-8L in E1A-induced apoptosis was further supported by the fact that procaspase-8L DN mutants inhibited caspase-8 activity and cell death. E1A-induced cleavage of procaspase-8L was normal in FADD-null cells but sensitive to the caspase inhibitor zVAD-fmk, consistent with either FADD-independent auto-processing or cleavage by an upstream caspase. This cleavage was also inhibited by BCL-2, which might relate to observed interactions between BCL-2 and BAP31 (Ng et al., 1997; Ng and Shore, 1998) and potential functions for BCL-2 at the ER in regulating organelle-specific initiation of cell death (Ferri and Kroemer, 2001). The potential activation of caspase-8 at a site other than cell surface death receptors suggests an additional pathway for regulating this important initiator caspase, which could initiate a cascade of caspase activation by processing conventional procaspase-8/a,b (Stegh et al., 2000) and/or downstream effector procaspases, as well as to activate pro-apoptotic targets such as BID (Korsmeyer et al., 2000). Of note, BAP31 itself is a preferred substrate of caspase-8 and its cleavage contributes directly to apoptosis progression (Ng et al., 1997). Recruitment of procaspase-8L to the BAP complex, therefore, may also place the enzyme at an important site of action for caspase-8.

2.6 Acknowledgements

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Chapter 3

Caspase-Resistant BAP31 Inhibits Fas-Induced Membrane Blebbing and Release of Cytochrome c from Mitochondria

3.1 Rationale

While conducting the experiments in Chapter 2, we noted that stable expression of crBAP31 exhibited a modest protection against E1A-induced apoptosis, suggesting that caspase cleavage of BAP31 might contribute to the progression of apoptosis in some manner. However, crBAP31 cells did not show any difference in the kinetics of procaspase-8L processing compared to parental cells or cells stably expressing EGFP, and therefore, crBAP31 did not seem to exert this protective effect at the level of procaspase-8L activation. Given that BAP31 is a known caspase-8 substrate (Ng et al., 1997), that the p20 caspase-cleavage product of BAP31 is an inducer of apoptosis, and that other caspase-8 substrates are known to play key signaling roles during the initiation phase of death receptor induced apoptosis (Li et al., 1998; Stegh et al., 2000), it is possible that BAP31 cleavage also plays a role in such pathways. The mechanism by which crBAP31 conferred protecting from cell death was further explored in Chapter 3, which is divided into two sections that were published as individual manuscripts. The aim of the first section was to pinpoint exactly what features of apoptosis crBAP31 inhibited, in the hope of gaining insight into what BAP31 cleavage normally accomplishes. The Fas cell death pathway was chosen for these studies because caspase-8 is activated by the Fas receptor complex at the plasma membrane. Since caspase-8 activation occurs independently of the BAP31 complex at the ER, BAP31 can be studied purely as caspase-8 substrate on this pathway. Fas signaling is particularly suitable for studying the role of caspase-8 substrates because, unlike the E1A pathway (Breckenridge and Shore, 2000), caspase-8 plays a nonredundant role at the apex of this pathway (Varfolomeev et al., 1998). Therefore, the signaling roles of caspase-8 substrates like BAP31 may be best dissected in

the Fas pathway. In the second section of this chapter, we sought to gain insight into BAP31 function by undertaking proteomic analysis of the BAP31 complex.

3.2 Abstract

BAP31 is a 28 kDa integral membrane protein of the endoplasmic reticulum whose cytosolic domain contains two identical caspase recognition sites (AAVD.G) that are preferentially cleaved by initiator caspases, including caspase-8. Cleavage of BAP31 during apoptosis generates a p20 fragment that remains integrated in the membrane and, when expressed ectopically, is a potent inducer of cell death. To examine the consequences of maintaining the structural integrity of BAP31 during apoptosis, the caspase-recognition aspartate residues were mutated to alanine, and Fas-mediated activation of caspase-8 and cell death were examined in human KB epithelial cells stably expressing the caspase-resistant mutant. crBAP31 only modestly slowed the time course for activation of caspases, as assayed by processing of procaspases -8 and -3 and by the appearance of total DEVDase activity. As a result, cleavage of the caspase targets poly(ADP-ribosyl) polymerase and endogenous BAP31, as well as redistribution of phosphatidylserine and fragmentation of DNA, were observed. In contrast, cytoplasmic membrane blebbing/fragmentation and apoptotic redistribution of actin were strongly inhibited, the cells retained a near normal morphology, and irreversible loss of cell growth potential following removal of the Fas stimulus was delayed. Of note, crBAP31 cells also resisted Fas-mediated release of cyt.c from mitochondria, and the mitochondrial electrochemical potential was only partly reduced. These results argue that BAP31 cleavage is important for manifesting cytoplasmic apoptotic events associated with

membrane fragmentation, and reveal an unexpected cross-talk between mitochondria and endoplasmic reticulum during Fas-mediated apoptosis in vivo.

3.3 Introduction

Programmed cell death is characterized by a series of morphological and structural changes culminating in the coordinated packaging of cellular contents into apoptotic bodies, which are ultimately eliminated following phagocytosis by neighboring cells. Early events in this process typically include cell rounding, loss of phospholipid asymmetry in the cell membrane, extensive cytoplasmic membrane blebbing and fragmentation, nuclear pyknosis, and internucleosomal DNA cleavage (Mills et al., 1999). Although much remains to be learned about the mechanisms underlying these events, it is achieved in most cell death pathways as a consequence of the proteolytic cleavage of a diverse array of structural and regulatory proteins by the executors of apoptosis, the caspase family of cysteine proteases (Earnshaw et al., 1999; Nicholson, 1999; Zheng et al., 1999). Several of these caspase targets have now been assigned critical roles in at least some of the apoptotic processes. They include the DFF40/CAD inhibitor, DFF45/ICAD, for fragmentation of DNA (Liu et al., 1997) and the actin-associated capping protein, gelsolin, for cytoplasmic membrane blebbing (Kothakota et al., 1997). As well, activation of several kinases by caspase cleavage, including PAK2 (Lee et al., 1997) and the Ste20-related kinases, MST1 (Graves et al., 1998) and SLK (Sabourin et al., 2000), contribute to loss of focal adhesions and retraction/disassembly of actin stress fibers, events that are associated with elaborate changes to the actomyosin network and membrane remodeling (Mills et al., 1999).

In contrast to many death-stimulating pathways, the proximal molecular events following activation of CD95/Fas with either Fas ligand or agonistic anti-Fas antibody are well understood. Receptor stimulation results in the assembly of a death inducing signaling complex that includes the adaptor protein, FADD, and procaspase-8 (Medema et al., 1997; Muzio et al., 1996). The resulting activation of this initiator caspase ultimately leads to processing of effector procaspases, including caspase-3, and apoptosis. A cytosolic target of caspase-8, proapoptotic BID, is cleaved and the truncated product, tBID, translocates to mitochondria where it stimulates release of intermembrane proteins, including cytochrome c (Li et al., 1998; Luo et al., 1998). BID appears to be a critical effector of this pathway, at least in certain contexts, since death receptor-induced redistribution of cytochrome c was not observed in *Bid*$^{-/-}$ mouse thymocytes and hepatocytes (Yin et al., 1999). Released cytochrome c in turn contributes to activation of initiator caspase-9, which in many death pathways is important for subsequent processing of downstream effector procaspases (Budihardjo et al., 1999). In contrast, Fas stimulation of certain cell types activates high levels of caspase-8 that are sufficient to process effector procaspases directly, whereas other cell types, at least in culture, activate low levels of procaspase-8 and likely depend on mitochondrial events for amplification of the caspase cascade (Scaffidi et al., 1998; Scaffidi et al., 1999).

Here, we show that human epithelial cells expressing a caspase-resistant mutant of BAP31, a preferred substrate for initiator caspases –8 (Ng et al., 1997), are resistant to a number of cytoplasmic changes that typically occur during Fas-mediated apoptosis. BAP31 is a 28 kDa integral membrane protein that is ubiquitously expressed (Adachi et al., 1996; Mosser et al., 1994) and highly enriched in the ER (Annaert et al., 1997; Ng et al., 1997) where it forms a homo-oligomer (Ng and Shore, 1998). The protein contains

three predicted transmembrane segments within the amino-terminal half of the protein that confer a topology in the ER membrane in which the short hydrophilic amino-terminus and an approximately 37 amino acid loop connecting TM2 and TM3 face the ER lumen, leaving a 14 kDa domain, terminating in a canonical KKXX ER retention signal, exposed to the cytosol (Ng et al., 1997). The cytosolic tail also contains a predicted weak death effector and overlapping coiled-coil (DECC) domain, flanked on either side by identical caspase recognition sites. These sites are cleaved in response to diverse death stimuli in vivo (Granville et al., 1998; Ng et al., 1997) and, in vitro, are preferred by caspases –8 and –1 and only weakly cleaved by effector caspase-3 (Ng et al., 1997). The resulting membrane-integrated p20 BAP31 fragment, when expressed ectopically, is a potent inducer of apoptosis, suggesting that BAP31 cleavage may contribute in some manner to the death process. Moreover, the influence of BAP31 may also extend to a role in regulating caspases, a suggestion consistent with the observation that BAP31 can associate with procaspase-8, BCL-2, and CED-4 in co-transfected cells (Ng et al., 1997; Ng and Shore, 1998). To investigate the contribution of BAP31 cleavage to apoptosis, therefore, we have created cell lines expressing a caspase-resistant (cr) mutant form of the protein, and examined its influence on cell death following independent stimulation of caspase-8 activity by the Fas signaling complex.

3.4 Materials and Methods

Plasmids and transfectants - cDNA encoding human BAP31, containing the Flag peptide epitope inserted between amino acids 242 and 243, was incorporated into the pcDNA3.1 expression vector, as previously documented (30). Site directed mutagenesis was

performed to convert aspartate residues at positions 164 and 238 to alanine, and authenticity of the crBAP31-Flag mutant expression vector confirmed by DNA sequence analysis. pcDNA3.1 vectors expressing CrmA (gift from V. Dixit), BAP31-Flag, and crBAP31-Flag were stably expressed in human KB epithelial cells, as described (30).

Antibodies and immunoblots - The following antibodies were employed: Mouse M2 anti-Flag (Sigma); chicken anti-human BAP31 (Ng et al., 1997); rabbit antibodies against the catalytic subunits of human caspase-8 (gift from D. Nicholson) and caspase-3 (gift from R. Sékaly); mouse anti-PARP (Biomol); mouse monoclonal 2G8.B6 and 7H8.2C12 antibodies against cytochrome c (gift from R. Jemmerson); and rabbit anti-γ-actin (gift from P. Braun). Cell extracts containing equivalent amounts of protein were resolved by SDS PAGE, the proteins transferred to nitrocellulose, incubated with the indicated antibody, and visualized with secondary antibody coupled to enhanced chemoluminescence.

Immunofluorescence - Cells were fixed in 4% paraformaldehyde, and incubated with mouse monoclonal 2G8.B6 anti-cytochrome c followed by goat anti-mouse IgG conjugated to Texas Red or with anti-γ-actin and goat anti-rabbit IgG coupled to Alexa 488 (Molecular Probes). Cells were visualized by fluorescence confocal microscopy.

Electron microscopy - Cells were treated with anti-Fas antibody for the indicated times. After two washes in PBS, cell pellets were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate for 2 h, washed, treated for 1 h in 0.1% osmium tetroxide, and finally dehydrated in acetone. The pellet was infiltrated with epon-acetone and embedded. Thin

sections (approx 100 nm; stained with 4% uranyl acetate / lead citrate) and thick sections (approx 0.5 μM; stained with toluidine blue) were examined by electron or conventional light microcopy, respectively.

Apoptosis assays - Human KB epithelial cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum and treated at approx. 80% confluency with mouse activating anti-Fas antibody (Upstate Biotechnology) and 10 μg/ml cycloheximide. At the indicated times, cells were collected and analyzed. Cell viability and the structural integrity of the plasma membrane was measured by the ability of cells to exclude trypan blue, as determined microscopically. For assessing phosphatidylserine redistribution and mitochondrial transmembrane potential, cells were incubated for 15 min at 37^0C in PBS containing 2% fetal bovine serum and 40μM $DiOC_6$ (Molecular Probes) or 0.1 μM fluorescein-conjugated human annexin V (R & D). Following two washes in the same medium, fluorescence was measured by flow cytometry. Caspase activity in whole cell extracts was obtained by treating cells with 50 mM Hepes, pH7.4, 1% Triton X-100, 5 mM EDTA, and 2 mM dithiothreitol, and incubating the extract with 50 μM AcDEVD-amc for 30 min at 37^0. Fluorescence in the linear range of DEVDase activity was determined using a plate reader (Tecan).

Cytochrome c. release - Cells (4×10^6) were washed in PBS and suspended in 0.1 ml 200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.5. After one cycle of freeze and thaw, the cells were homogenized with 35 strokes in a motorized Teflon-glass homogenizer operating at 2000 rpm, and centrifuged at 800 x g for 10 min to remove

nuclei and cell debris.. The supernatant was centrifuged at 100,000 x g for 10 min, and the membrane pellet resuspended in homogenization buffer to the same volume as the 100,000 x g supernatant. Equivalent volumes of the pellets and supernatants were subjected to SDS PAGE and immunoblotting with mouse monoclonal 7H8.2C12 anti-cytochrome c.

3.5 Results

3.5.1 Caspase-resistant BAP31

The schematic in Fig. 3.1 shows the predicted topology of BAP31 in the ER membrane and the location of the caspase recognition sites (AAVD.G) flanking the DECC domain in the cytosolic tail (Ng et al., 1997). Treatment of human KB epithelial cells with agonistic mouse antibody against Fas (0.5 μg/ml), in the presence of 10 μg/ml cycloheximide (CHX) to enhance sensitivity to Fas activation (Scaffidi et al., 1999), resulted in cleavage of the 246 amino acid full length BAP31, generating two products of approximately 27 and 20 kDa (Fig. 3.1A). These corresponded to cleavage at aspartates 238 and 164, respectively (Ng et al., 1997). Caspase-resistant (cr) BAP31 was produced by mutating these residues to alanine, and both this construct and that encoding wild-type BAP31 were further manipulated to include Flag epitope inserted immediately upstream of the ER retention signal, KKEE, located at the extreme carboxy-terminus of the protein. KB cell lines stably expressing wt BAP31 or crBAP31 were then examined by immunoblotting with anti-Flag antibody. crBAP31-Flag, but not BAP31-Flag, was found to remain structurally intact in the face of sustained stimulation with anti-Fas (Fig.

3.1B). In both cases, the proteins were expressed at approximately 3-times the level of endogenous BAP31, as assessed using a BAP31 polyclonal antibody (not shown).

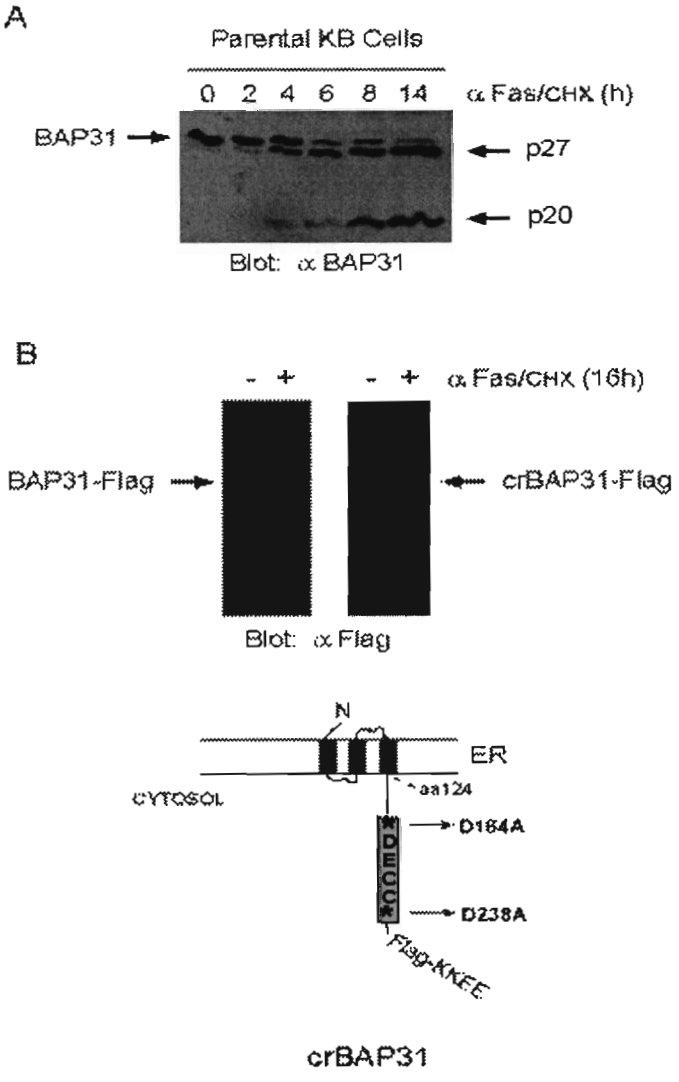

Figure 3.1. Human KB epithelial cells expressing crBAP31. (A) Control (parental) KB cells were stimulated with 0.5 μg/ml anti-Fas antibody in the presence of 10 μg/ml cycloheximide (CHX) for the indicated times, and whole cell lysates were subjected to SDS PAGE, immunoblotted with chicken anti-BAP31, and visualized by enhanced chemoluminescence (30). The positions of full length BAP31 and the BAP31 caspase-cleavage products, p27 and p20, are indicated. (B) As in A except that KB cells stably expressing wt BAP31-Flag or crBAP31-Flag were analyzed with mouse anti-Flag antibody. (C) Schematic of crBAP31-Flag in the endoplasmic reticulum (ER), in which the caspase-recognition aspartate (asterisk) residues at positions 164 and 238 have been mutated to alanine. The Flag epitope tag was inserted immediately upstream of the COOH-terminal tetra-peptide ER retrieval signal, KKEE.

3.5.2 crBAP31 inhibits Fas-mediated apoptotic membrane blebbing and fragmentation.

Control (parental) KB epithelial cells and KB cells stably expressing crBAP31-Flag or the cowpox serpin, CrmA, were stimulated for 16 h with anti-Fas and examined by staining with trypan blue to assess the integrity of the plasma membrane (Fig. 3.2A) and by immunoblotting with antibody against the catalytic subunit of caspase-8 to assess procaspase-8 processing (Fig. 3.2B). crBAP31 had little influence on Fas-mediated processing of procaspase-8, as judged by loss of the 54-55 kDa forms of procaspase-8. As expected, CrmA, an inhibitor of caspase-8 that prevents activation of procaspase-8 in vivo (Garcia-Calvo et al., 1998), significantly retarded procaspase-8 processing (Fig. 3.2B). Despite the fact that crBAP31 had little or no influence on procaspase-8 processing in response to Fas stimulation, however, crBAP31 was equally as effective as CrmA at blocking the loss of cellular integrity, as visualized by staining of cells with trypan blue (Fig. 3.2A). These findings were extended and quantified by examining the appearance of the cells by electron microscopy (Fig. 3.3). After stimulation by Fas antibody, control cells exhibited extensive membrane blebbing and formation of apoptotic bodies; crBAP31 cells, on the other hand, remained intact (Fig. 3.3A). Numerical scoring of these cells in thick sections stained with toluidine blue revealed that greater than 60% of control cells had acquired this extreme membrane remodeling by 7 h post-stimulation of Fas, and this extended to greater than 90% by 16 h post-stimulation (Fig. 3.3B). This was inhibited by crBAP31 (Fig. 3.3B), to an extent similar to that observed for CrmA-expressing cells (not shown). Of note, no difference in this or other assays was observed between control cells and cells expressing wt BAP31-Flag, indicating that the observed effects of crBAP31-Flag, which was expressed at similar levels as wt BAP31-Flag, was

not due to the fact that these cells carried an approximately 3-fold excess of the BAP31 protein.

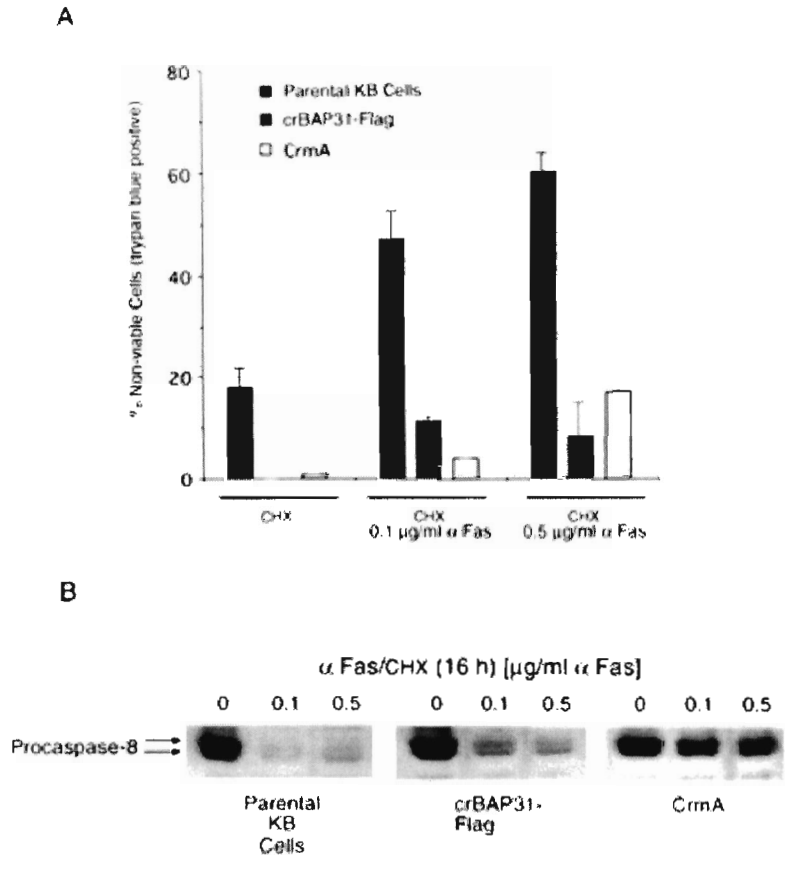

Figure 3.2. crBAP31 permits Fas-mediated activation of caspase-8 but inhibits the loss of plasma membrane integrity. (A) Control (parental) KB cells or KB cells stably expressing crBAP31-Flag or CrmA were stimulated with anti-Fas/CHX or CHX alone for 16 h, and the percentage of the cells that were stained with trypan blue was determined. (B) As in A except that total cell lysates were subjected to SDS PAGE and immunoblotted with rabbit antibody raised against the p18 catalytic subunit of human procaspase-8. The positions of procaspase-8/a/b are indicated.

The early stages of apoptotic membrane remodeling has been associated with actin redistribution to the cell periphery during cell rounding (Mills et al., 1999). A

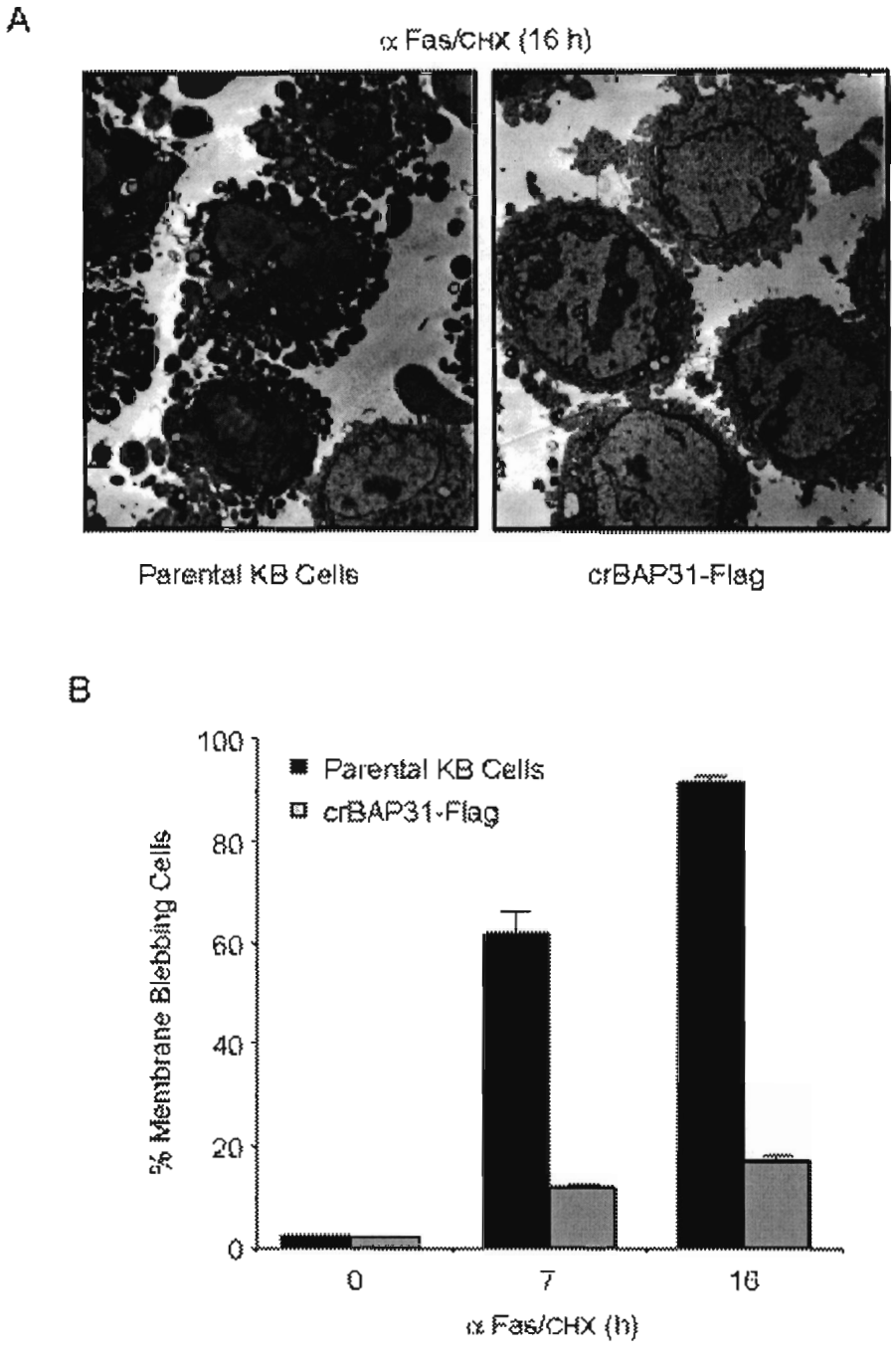

Figure 3.3. crBAP31 inhibits Fas-mediated apoptotic membrane blebbing and fragmentation. (A) Transmission electron microscopy of Fas-stimulated control (parental) KB epithelial cells and KB cells stably expressing crBAP31-Flag. (B) The number of apoptotic membrane blebbing cells was scored in thick sections and expressed as a percent of total cells. Averages of 3 independent determinations and standard deviations are presented.

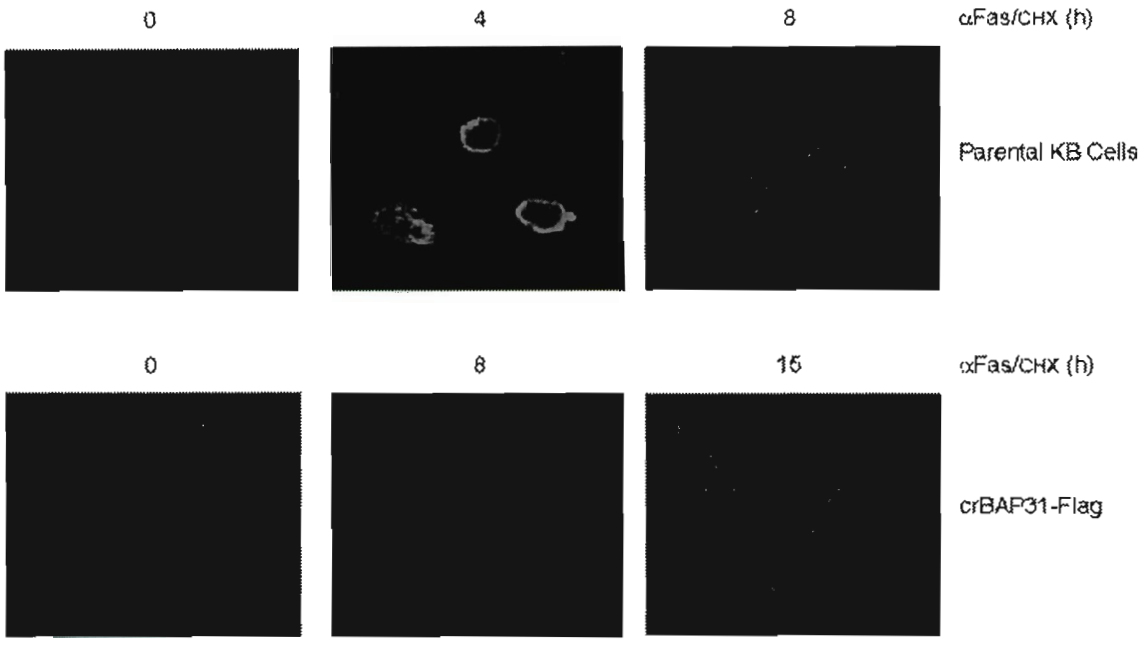

Figure 3.4. crBAP31 inhibits Fas-mediated redistribution of γ-actin. Control (parental) KB epithelial cells and KB cells stably expressing crBAP31-Flag were stimulated with anti-Fas/CHX for the indicated times and examined by immunofluorescence confocal microscopy using rabbit anti-γ-actin and goat anti-rabbit IgG coupled to Alexa 488. Representative images are shown.

similar redistribution of γ-actin was observed 4 h after Fas stimulation of control KB epithelial cells, whereas crBAP31 cells maintained a normal distribution of γ-actin and the cells remained flat and adherent even up to 15 h post-stimulation (Fig. 3.4). Collectively, these findings reveal that crBAP31 has a strong inhibitory influence on both the cell shape / cytoskeletal changes and remodeling of membranes into the blebbs and vesicular bodies that are characteristic of apoptosis.

3.5.3 Activation of caspases in cells expressing crBAP31.

To assess the presence of caspase activity in extracts from control cells and from cells expressing crBAP31 or CrmA, equivalent amounts of extract protein were incubated

with the fluorogenic peptide, DEVD-amc, which is a general substrate for effector caspases -3 and –7 (Garcia-Calvo et al., 1998). The presence of crBAP31 appeared to delay the induction of DEVDase activity in response to Fas stimulation, but by 15 h levels were similar to that recorded for control cells (Fig. 3.5A). In contrast, cells expressing CrmA significantly inhibited the appearance of DEVDase, but did not reduce activity to baseline, which was established in control cell extracts using the non-cleavable peptide inhibitor, DEVD-fmk (Fig. 3.5A). This may indicate that the expression level of CrmA in these cells was insufficient to completely abolish all activation of caspase-8 in response to Fas stimulation or that a small amount of DEVDase activity arises in these cells independently of casapase-8 initiation. The retardation of appearance of DEVDase activity in the presence of crBAP31 was reflected in a slower time course for Fas-stimulated processing of procaspase-3 in crBAP31 cells compared to controls (Fig. 3.5C), but this was insufficient to significantly influence cleavage of the caspase –3 and –7 substrate, poly(ADP-ribosyl) polymerase (PARP) (Fig. 3.5D). Of note, caspase cleavage of endogenous BAP31 in the crBAP31 cells also was observed (Fig. 3.5B).

Other hallmark features of apoptosis that are a consequence of caspase activity include the acquisition of annexin V staining due to caspase-induced redistribution of phosphatidyl serine to the outer aspect of the plasma membrane and DNA fragmentation resulting from caspase-dependent inactivation of the inhibitor of DFF40/CAD, DFF45/ICAD (Green, 2000; Liu et al., 1997). Annexin V staining and fragmentation of DNA were both observed in crBAP31 cells and by 16 h post-stimulation of Fas they occurred to an extent similar to that in control cells (Fig. 3.5E,F). The sustained inhibition of apoptotic cell morphology that is conferred over this time period by the

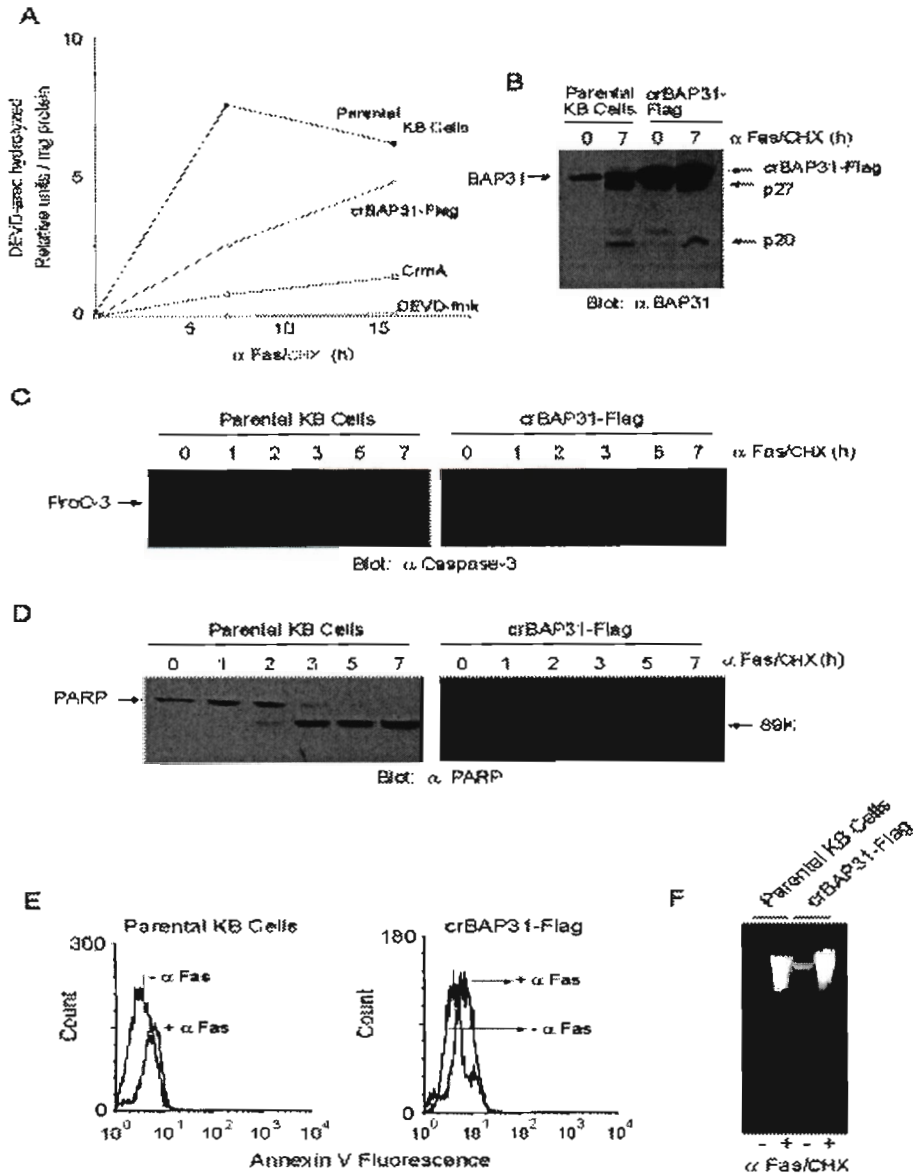

Figure 3.5. Fas-mediated activation of caspases in crBAP31-expressing cells. (A) Total cell lysates were prepared from control (parental) KB epithelial cells or from KB cells stably expressing crBAP31-flag or CrmA, and equivalent, rate-limiting amounts of protein were assayed for cleavage of DEVD-amc, during which the resulting fluorescence was detected and quantified in the linear time-course range using an automated fluorescence plate reader detecting 460 nm wavelength. Control cell lysate was also assayed in the presence of 1 μM of the inhibitor, DEVD-fmk. An average of two independent determinations is presented. (B) As in Fig. 1, control (parental) and crBAP31-Flag-expressing KB cells were subjected to SDS and immunoblotting with chicken anti-Bap31 under the conditions indicated. The positions of full length proteins and the p27 and p20 cleavage products of BAP31 are indicated. (C) As in B except that immunoblots were developed using rabbit anti-caspase-3. (D) Immunoblots were developed using mouse monoclonal antibody against poly(ADP-ribosyl) polymerase (PARP), and the 89 kDa caspase cleavage product denoted . (E) Cells were treated with or without anti-Fas/CHX for 16 h, stained in situ with Annexin V, and fluorescence intensity determined by FACS analysis. (F) As in E except that low molecular weight DNA was isolated, resolved by agarose gel electrophoresis, and stained with ethidium bromide. The intense staining fragment migrating toward the top of the gel has an approximate size of 25 kbp.

presence of crBAP31 following Fas stimulation (Fig. 3.3 and 3.4), therefore, occurs in the face of activated caspases, cleavage of the caspase targets, PARP and endogenous BAP31, redistribution of phosphatidyl serine in the plasma membrane, and fragmentation of nuclear DNA.

3.5.4 Influence of crBAP31 on Fas-induced transformations of mitochondria.

Release of cyt.c from the mitochondrial inter-membrane space is a common response to many death signals, and typically occurs at an early step in the apoptotic death pathway (Goldstein et al., 2000). Fig. 3.6A presents confocal microscopic images of control and crBAP31-expressing KB epithelial cells before and after stimulation with activating anti-Fas antibody for 7 h. Fas stimulation caused cyt.c to redistribute from a mitochondrial location to a diffuse pattern throughout the cytoplasm. Of note, this redistribution of cyt.c was observed in intact, flat, adherent cells prior to acquisition of the condensed, membrane-fragmented apoptotic morphology (arrow in Fig. 3.6A), consistent with release occurring early in the Fas-mediated death pathway. Strikingly, cells expressing crBAP31 resisted cyt. c redistribution in response to Fas stimulation (Fig. 3.6A), and this finding was extended to an analysis of total cell populations by fractionation of cell homogenates and immunoblotting (Fig. 3.6B). In control cells prior to Fas stimulation, cyt. c was recovered in the membrane fraction containing mitochondria, and not in the high-speed supernatant fraction. Fas stimulation resulted in a progressive increase in recovery of cyt. c in the high-speed supernatant, reaching an apparent maximum by about 8 h. Again, crBAP31 cells largely retained cyt.c in the mitochondrial fraction, with a constant and low level amount recovered in the high-speed supernatant throughout the time course of Fas stimulation up to 16 h (Fig. 3.6B).

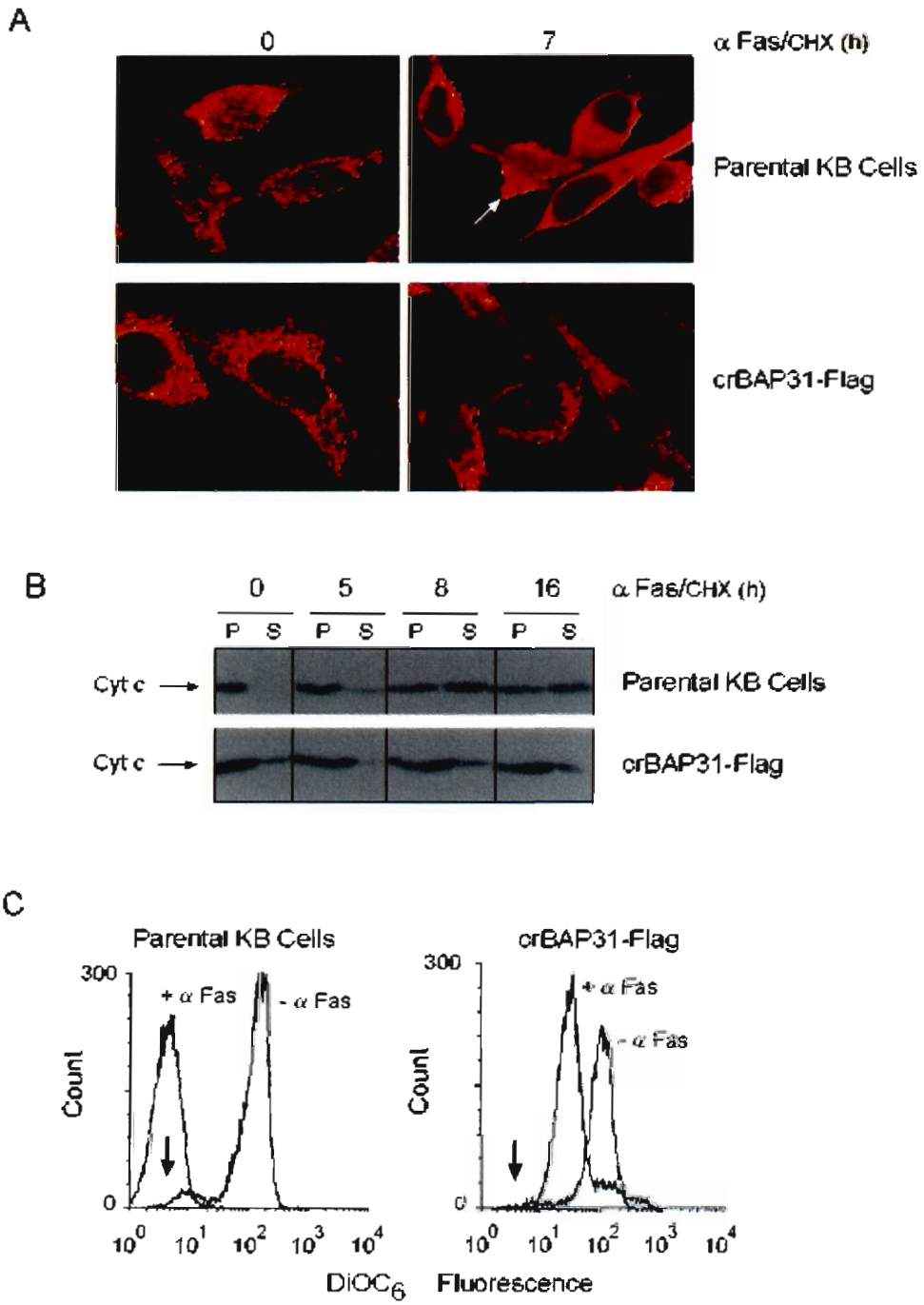

Figure 3.6. crBAP31 inhibits Fas-mediated release of cyt.c from mitochondria. (A) Control (parental) KB epithelial cells and KB cells stably expressing crBAP31-Flag were stimulated with anti-Fas for the indicated times and examined by immunofluorescence confocal microscopy using anti.cyt. c antibody. Release from mitochondria can be observed in cells prior to membrane blebbing (arrow denotes an obviously apoptotic cell). (B) At the indicated times of treatment, cells were homogenized and the post-nuclear supernatant separated into membranes (100,000 x g pellet) (P) and supernatant (S), as indicated, and equal aliquots subjected to SDS PAGE and immunoblotting with 7H8.2C12 anti-cyt.c (cyt c). (C) Cells were treated with or without anti-Fas for 16 h, stained with the mitochondrial potential-sensitive dye, DiOC6, and subjected to FACS analysis. The arrow denotes the peak of fluorescence intensity obtained for cells in which the mitochondrial electrochemical potential was collapsed following treatment with 1 μM carbonylcyanide *m*-chlorophenyl hydrazone (CCCP).

In addition to cyt.c release from mitochondria, the organelle also undergoes other transformations in response to apoptotic stimuli, including a collapse of the transmembrane electrochemical potential at the inner membrane (inside negative). The status of the mitochondrial electrochemical potential in control and crBAP31 cells with and without Fas stimulation was monitored using the potential sensitive dye, $DiOC_6$, and FACS analysis (Fig. 3.6C). Whereas control cells exhibited a collapse of the transmembrane potential after Fas stimulation for 16 h, equivalent to treatment with the protonophore CCCP (arrow in Fig. 3.6C), crBAP31 cells responded with a lower but retained potential, indicating that mitochondria in these cells remained at least partly polarized.

3.5.5 Recovery of cell growth potential following removal of the Fas stimulus .

In Fig. 3.7, control KB cells and KB cells expressing either crBAP31 or CrmA were treated for 7 h with anti-Fas, the cells were collected following trypsinization, washed, and then equivalent numbers of cells were examined for their ability to attach to culture plates and grow in fresh media lacking the Fas stimulus. Neither CrmA nor crBAP31 conferred any growth difference to these cells in the absence of external treatments (not shown). However, whereas treatment of control cells with anti-Fas resulted in a low number of cells recovering and growing on cell plates by 4 days after the removal of the Fas death stimulus, greater than 20x this number was recorded for cells expressing crBAP31 and about 50x this number recorded for CrmA-expressing cells. Of note, after 7 h stimulation by anti-Fas, most of the full length PARP in crBAP31 cells was typically cleaved to the 89 kDa apoptotic fragment (Fig. 3.5D). These results demonstrate, therefore, that a significant number of crBAP31 cells could at least delay the

irreversible loss of cell growth potential that was experienced by control cells in response to Fas stimulation, despite the manifestation of caspase activity.

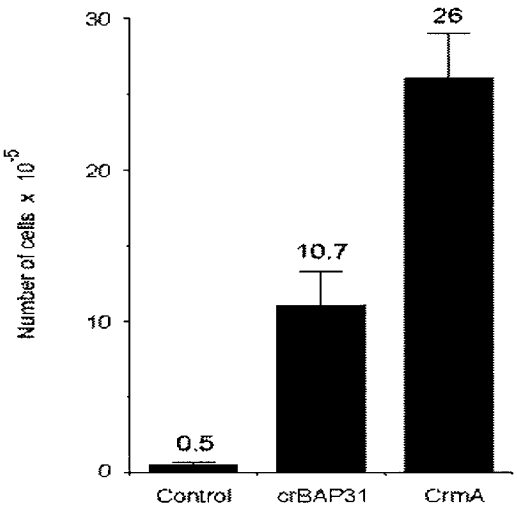

Figure 3.7. crBAP31 inhibits the loss of cell growth potential in response to Fas stimulation. 2 x 10^5 of the indicated cells were seeded in 12 well dishes overnight, stimulated with anti-Fas for 7 h, and total cells collected after trypsinization, washed twice in PBS, and plated onto 100 mm plates. After 4 days, cell counts were taken. Shown are the average counts from 3 independent measurements (numerical values above the bars) and standard deviations.

3.6 Discussion

BAP31 is highly enriched in the ER as judged by quantitative cryo-immunocytochemistry employing proteinA-gold and electron microscopy (our unpublished data). Interestingly, however, BAP31 has been reported to form associations with distal constituents in the secretory pathway, including IgD (Adachi et al., 1996) and cellubrevin (Annaert et al., 1997), observations that suggest that BAP31 may play a role in the egress of at least certain proteins out of the ER. Its potential role in apoptosis, on

the other hand, derived from the finding that BAP31 can associate with BCL-2, which also localizes to the ER (Krajewski et al., 1993), and is a target for caspases (Ng et al., 1997). Moreover, BAP31 was found to form a complex with procaspase-8 and the *C. elegans* caspase adaptor, CED-4, in co-transfected cells, suggesting that the BAP31 complex may play a role in regulating caspase activity (Ng et al., 1997; Ng and Shore, 1998). Here, we have chosen to investigate BAP31 as a target of caspases, by independently activating caspases via the Fas signaling complex in cells that stably express crBAP31. BAP31 is efficiently cleaved by caspase-8 and related caspases, generating a p20 product that remains integrated in the membrane and, when expressed ectopically, is a potent inducer of apoptosis (Ng et al., 1997). We have noted in diverse circumstances, however, that p20 readily complexes with epitope-tagged full length BAP31 (Ng and Shore, 1998). In the present study, therefore, it is not known if the effects of crBAP31 are due to preserving the structural integrity of the protein (preventing loss of BAP31 function) or due to interference with p20BAP31 proapoptotic activity by sequestering the cleaved molecule (preventing p20 gain of function).

Activation of caspase-8 is an important proximal event in the CD95/Fas cell death pathway (Medema et al., 1997; Varfolomeev et al., 1998), and the ability of caspase-8 to initiate a caspase cascade, either directly or via amplification of mitochondrial-dependent intermediate steps, provides the mechanism for cellular execution by apoptosis (Scaffidi et al., 1998; Scaffidi et al., 1999; Stegh et al., 2000). One target of caspase-8 in the Fas pathway, proapoptotic BID, is an important and, at least in certain contexts, essential (Yin et al., 1999) effector of cyt. c release from mitochondria (Li et al., 1998; Luo et al., 1998). Cleavage of BID by caspase-8 or other caspases results in removal of an inhibitory NH_2-terminal segment, rendering the BH3 domain of the truncated product, tBID, available for

interaction with partner proteins (Wang et al., 1996). We demonstrate here, however, that another target of caspase-8 (or other caspase) in the Fas pathway, BAP31, may also play an important role both in cytochrome c release from mitochondria and in the extensive membrane remodeling that is associated with blebbing and formation of apoptotic bodies during the cytoplasmic execution phase of cell death. Interestingly, we observe no inhibition of BID cleavage in Fas-stimulated KB cells expressing crBAP31 (data not shown). Thus, while several studies have shown that incubation of mitochondria with caspase-8-treated naïve cytosolic extracts or with tBID alone can induce egress of cytochrome c from the organelle in vitro (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998), the present results argue that the cellular environment or other factors related to the ER may be critical for these events to manifest in vivo. Maintaining the structural integrity of BAP31 in the ER even in the face of sustained caspase activity may preclude these collateral cellular changes from occurring in the Fas death pathway. Emerging evidence, for example, suggests that BID may cooperate with BAX (or BAK) to stimulate cytochrome c release from mitochondria (Desagher et al., 1999), and that a conformational change in BAX in response to a death signal is important for insertion of BAX into the mitochondrial outer membrane (Eskes et al., 2000; Goping et al., 1998). While cytosolic factors such as BID and tBID can induce such conformational changes in BAX in vitro (Eskes et al., 2000), additional considerations may apply in vivo. One report for example indicates that BAX activation can occur in response to death signal-induced changes in cellular pH (Matsuyama et al., 2000). It may be that BAP31 or its cleavage product influences this or other ER-regulated conditions that favor a cellular environment in which the proapoptotic activity of tBID and BAX/BAK can be realized.

Release of cyt.c from mitochondria is an early event in many death signaling pathways, and contributes to downstream activation of a caspase cascade. In the human KB epithelial cells studied here, Fas-mediated cyt.c release was not essential for effector caspase activation, since crBAP31 inhibited the former but not the latter. In this regard, these cells exhibited type I properties (Scaffidi et al., 1998; Scaffidi et al., 1999). Consequently, crBAP31 cells resisted the morphological changes associated with cytoplasmic apoptosis in the face of sustained activity of caspases. The various stages of cytoplasmic apoptosis - release from extracellular matrix attachments and reorganization of focal adhesions (rounding); plasma membrane changes associated with dynamic membrane blebbing; and finally membrane fragmentation and formation of apoptotic bodies (condensation) - are typically associated with changes in the organization of the cellular actomyosin complex and, especially in the later stages, this involves caspase-dependent cleavage of actomyosin-associated structural proteins, regulators, and signaling molecules (Mills et al., 1999). It may be noteworthy, therefore, that the cytosolic tail of BAP31, from leucine 122 to alanine 236, can be arranged within 4 segments of 4 heptads each in which the frequency of hydrophobic residues at the 1 and 4 positions is 71% (27), similar to that observed in myosin heavy chain coils (Mosser et al., 1994). Moreover, immunoprecipitation of BAP31-Flag and analysis of associated polypeptides by exhaustive tandem mass spectrometry has identified both γ-actin and non-muscle myosin II heavy chain B as proteins that may constitutively interact with the BAP31 complex in vivo (see below). These associations are lost for p20 BAP31 and, therefore, might contribute to the apoptotic cell morphology.

Finally, a limited number of proteins have been identified whose caspase-resistant mutants, like crBAP31, have relatively broad inhibitory influences on the ability of the cell to undergo cytoplasmic apoptosis. These include structural proteins such as gelsolin (Kothakota et al., 1997) and signaling molecules such as PAK2 (Rudel and Bokoch, 1997). Additionally, however, cells expressing crBAP31 exhibited a resistance against Fas-induced release of cytochrome c and collapse of the mitochondrial electro-chemical potential, which presumably preserves or delays the cell from acquiring irreversible mitochondrial dysfunction (Vander Heiden and Thompson, 1999). This may help to explain the ability of at least a fraction of these cells to have recovered from Fas stimulation for 7 h, whereas control cells did not (Fig. 3.7). Moreover, the identification of caspase-resistant mutants such as crBAP31 that have death-inhibiting influences may be relevant to recent suggestions that caspases can play roles in physiological cell stimuli other than apoptosis. For example, cleavage of certain caspase targets but not of others has been noted during stimulation of T lymphocyte in the apparent absence of apoptosis (Alam et al., 1999). Collateral regulation that preserves the structural integrity of certain key caspase substrates, including BAP31, DFF45/ICAD, gelsolin, etc., might provide the mechanistic basis for this lack of apoptosis.

3.7 Acknowledgements

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The Resident Endoplasmic Reticulum Protein, BAP31, Associates With γ-Actin and Myosin B Heavy Chain: Analysis by Capillary Liquid Chromatography Microelectrospray Tandem Mass Spectrometry

3.8 Abstract

BAP31 is a 28-kDa integral membrane protein of the endoplasmic reticulum whose cytosolic domain contains two caspase recognition sites that are preferentially cleaved by initiator caspases, such as caspase-8. Recently, we reported that the caspase-resistant BAP31 inhibited Fas-mediated apoptotic membrane fragmentation and the release of cyt. c from mitochondria in KB epithelial cells (Nguyen M., Breckenridge G., Ducret A. and Shore G. (2000) Mol. Cell. Biol. *20*, 6731-6740). We describe here the characterization by capillary liquid chromatography microelectrospray tandem mass spectrometry of a BAP31 immunocomplex isolated from a HepG2 cell lysate in the absence of a death signal. We show that BAP31 specifically associates with non-muscle myosin heavy chain B and non-muscle γ-actin, two components of the cytoskeleton actomyosin complex. Collectively, these data confirm that BAP31, in addition to its potential role as a chaperone, may play a fundamental role in the structural organization of the cytoplasm. Here we also show that Fas stimulation of apoptosis releases BAP31 associations with these motor proteins, a step that may contribute to extranuclear events, such as membrane remodeling, during the execution phase of apoptosis.

3.9 Introduction

Apoptosis, or programmed cell death, is a physiological mechanism by which multicellular organisms can eliminate in an orderly fashion unwanted or damaged cells during development, maturation or reparation (Nicholson, 1999). Central to the trigger of

the apoptotic pathway is the activation of a family of cysteine proteases, the caspases, which have been shown to (in)activate a relatively large panel of proteins involved in essential physiological functions. Cumulatively, these proteolytic events disable homeostatic and repair processes, halt cell cycle progression, mediate structural disassembly and morphological changes, and mark the dying cell for engulfment and elimination.

Recently, we have identified a Bcl-2/Bcl-X_L and procaspase-8 associated protein, BAP31, a 28 kDa integral membrane protein resident in the endoplasmic reticulum (ER) of most if not all cell types (Adachi et al., 1996; Mosser et al., 1994; Ng et al., 1997). Sequence analysis reveals that BAP31 can be roughly divided in two domains (Fig. 1): a hydrophobic 15-kDa NH_2-terminal fragment is predicted to form three transmembrane domains in which the short hydrophilic NH_2-terminus and a 37 aa loop face the ER's lumen. The remaining 13-kDa domain, terminated by the canonical KKXX ER localization signal, is exposed to the cytosol (Ng et al., 1997). Functionally, BAP31 has been suggested to represent an ER-associated chaperone as it was first detected associated with membrane-bound immunoglobulin in lysates of B lymphocytes (Kim et al., 1994). Consistent with this proposed role, BAP31 can form transient associations with newly-synthesized IgD and cellubrevin as they exit from the endoplasmic reticulum to the Golgi apparatus (Annaert et al., 1997) and it has been recently shown to participate in the quality control of the cystic fibrosis transmembrane conductance regulator folding (Lambert et al., 2001).

In addition, BAP31 has also been suggested to be involved in apoptotic programmed cell death. It is capable of selectively recruiting the procaspase-8 isoform, procaspase-8L, which promotes apoptosis. BAP31 also associates with anti-apoptotic Bcl-2 family proteins, which make direct contact with the membrane-associated NH_2-terminal region of BAP31 (Ng et al., 1997; Ng and Shore, 1998). In particular, BCL-2 has been demonstrated to block the cell death pathway induced by expression of the E1A oncogene (Ng et al., 1997; Nguyen et al., 1998). In the absence of BCL-2, however, cell death signaling leads to the activation of procaspase-8L and the resulting proteolytic cleavage of BAP31 at two identical caspase-8 recognition sites within its cytosolic tail, removing the procaspase-8/Ced-4 recruitment domain and generating a p20 membrane-bound fragment of BAP31 (Breckenridge et al., 2002; Ng et al., 1997). When expressed ectopically, p20 BAP31 causes dramatic membrane remodeling and is a potent inducer of cell death (Ng et al., 1997), while cytoplasmic membrane blebbing and fragmentation and apoptotic redistribution of actin were strongly inhibited in a cell line containing a caspase-resistant BAP31 (Nguyen et al., 2000). Interestingly, the cytosolic region from Leu 122 to Ala 236 can be arranged within 4 segments of 4 heptads each in which the frequency of hydrophobic residues at the 1 and 4 positions is 71% (Mosser et al., 1994), similar to that observed in myosin heavy chain coils (Strehler et al., 1986).

In this paper, we describe the characterization of a BAP31 immunocomplex isolated from a cell lysate in the absence of a death signal (Nguyen et al., 2000). Consistent with the above-mentioned motif, predicting interactions between BAP31 and myosin, we show that BAP31 specifically associates with non-muscle myosin B heavy chain and γ-actin. This suggests an additional role for BAP31 in the ER membrane

architecture, traffic, and/or cargo movement in normal cell physiology. Interestingly, the cleavage of BAP31 by caspase-8 releases the tethering to these motor proteins via BAP31, a step that may contribute to extranuclear events, such as membrane remodeling, during the execution phase of apoptosis (Mills et al., 1999; Nguyen et al., 2000). We also show that with extended Fas stimulation, these associations between full length BAP31 and γ-actin are lost even in the absence of BAP31 cleavage.

3.10 Materials and Methods

Biologicals - cDNA encoding human BAP31 with the Flag peptide epitope sequence inserted between the codons for amino acid 242 and 243 was incorporated into the pcDNA3.1 expression vector as documented in (Nguyen et al., 2000). BAP31-Flag was stably expressed in the human HepG2 cell line. The KB cell line expressing crBAP31-Flag and vectors, methodology, and the H1299 cells used for transient transfection have been described (Breckenridge et al., 2002; Ng et al., 1997; Nguyen et al., 2000).

Immunoprecipitation of the pre-apoptotic BAP31 complex – The pre-apoptotic BAP31 complex was immunoprecipitated as described in (Nguyen et al., 2000). Briefly, cells were washed in PBS and homogenized in 1 ml lysis medium per 10-cm culture plate (50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% v/v Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride). After centrifugation at 11,000 g, the supernatant was pre-cleared 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 was incubated with mouse M2 anti-Flag

antibody (IBI-A Kodak Co., New Heaven, CT) at 4 °C for 6–8 h, at which time 20 µl of a 1:1 slurry of a Protein G sepharose was added. After 1 h of incubation at 4 °C, the beads were removed, washed, and boiled in SDS electrophoresis sample buffer.

Electrophoresis and proteolytic digestion - The eluted immunocomplex was directly analyzed by SDS-PAGE using a 10 %T, 2.6 %C, acrylamide gel (15 cm × 30 cm × 1 mm). The sample was run at room temperature in a Hoefer SE620 gel apparatus for 15 h at 100 V using a 2x Laemmli running buffer (50 mM Tris(hydroxymethyl)aminomethane, 385 mM glycine, 0.2% w/v SDS). Protein bands were visualized by Coomassie Blue staining. Bands of interest were excised from the gel and proteins were in-gel digested following a published protocol (Hellman et al., 1995) modified as follows. Briefly, the acrylamide bands were chopped in 1 mm^3 pieces that were washed for 20 min first in 50% v/v acetonitrile, then in 50 mM ammonium bicarbonate (unbuffered), 50% v/v acetonitrile, and finally in 15 mM N-ethylmorpholine, 5 mM acetic acid, 50% v/v acetonitrile. The gel pieces were then dried for 30 min in a speed-vac to remove any remaining liquid. The dried polyacrylamide was then rehydrated with 20–30 µl of the trypsin digest solution (15 mM N-ethylmorpholine, 5 mM acetic acid containing 15 ng/µl sequence-grade trypsin [Promega, Madison WI, USA]) so that the liquid was completely absorbed in the gel pieces. Digestion was performed overnight at room temperature. Peptides were collected by extracting the acrylamide three times 20 min with 40–60 µl 60% v/v acetonitrile, 0.5% v/v formic acid. The collected fractions were combined and the peptides were dried down in a speed-vac and kept at -20 °C until use.

Peptide mapping by capillary liquid chromatography microelectrospray tandem mass spectrometry (LC-μESI-MS/MS) - Tryptic peptides were analyzed using a self-packed capillary column (0.1×120 mm, Magic-MS C_{18} packing material, Michrom BioResources Inc., Auburn CA, USA) coupled to a Finnigan MAT TSQ7000 mass spectrometer (Thermo Finnigan, San Jose CA, USA) using a microelectrospray interface operated at 1.2 kV. The nanoliter flow rate required by the capillary LC column (700 nl/min) was obtained by coupling a Magic microbore HPLC system (Michrom BioResources) with a pre-column high-pressure flow splitter from the same supplier. Samples were reconstituted in 25 μl of buffer A (2.5% v/v acetonitrile, 0.1% v/v formic acid, 0.005% v/v heptafluorobutanoic acid; buffer B was 80% v/v acetonitrile, 0.085% v/v formic acid, 0.005% v/v heptafluorobutanoic acid), centrifuged for 5 min at 14,000 g, and the supernatant was injected off-line onto a C_{18} precolumn cartridge (0.5 mm × 1 mm, LC Packings Inc., San Francisco CA, USA) at 5 μl/min. Peptides were eluted from the column using a linear gradient from 10 to 60 % buffer B in 20 min.

For unambiguous identification, eluting peptides were subjected to automated tandem mass spectrometric analysis by collision-induced dissociation essentially as described in (Ducret et al., 1998) with some minor modifications. Briefly, peptides were subjected to tandem mass spectrometric analysis if the ion current for a particular species exceeded a relative intensity of 200,000 counts. After analysis, the mass of the investigated species was recorded into a user table that prevented the re-analysis of the same ion until its intensity had decreased under a user-defined threshold. This modification was essential to analyze complex mixtures when several peptides were usually co-eluting in a chromatographic peak.

Database searching - Uninterpreted tandem mass spectra were correlated to protein databases using the program SEQUEST version C1 (Yates et al., 1995) essentially as described in (Ducret et al., 1998). For identification purposes, all tandem mass spectra were matched against a subset of the NCBI Genbank protein database (http://www.ncbi.nlm.nih.gov; non-redundant protein database release July 2, 2001) filtered with the word "human" (resulting in a database of approximately 67,000 entries). Automated SEQUEST identifications were performed using default parameters with the peptide and fragment tolerance set to 1.5 Da and 1.0 Da, respectively, and with methionine dynamically searched for the commonly found methionine sulfoxide derivative (+16 Da). Further characterization was achieved by manually excluding tandem mass spectra of poor quality, by restricting the database to the proteins that were identified in a first pass approach, and by changing the SEQUEST parameter file to account for post-translational modifications as indicated in the text.

3.11 Results

3.11.1 Immunoprecipitation of the pre-apoptotic BAP31 complex and preliminary characterization by SDS-PAGE

In a recent work (Nguyen et al., 2000), we reported on the characterization of a caspase-resistant (cr) BAP31 that inhibited Fas-mediated apoptotic membrane blebbing and fragmentation in KB epithelial cells. crBAP31-Flag, whose caspase recognition aspartate residues were mutated to alanine residues (Fig. 3.8), only modestly slowed down the time course for activation of caspases, as assayed by the processing of

procaspases 8 and 3, by the measurement of total DEVDase activity, and by the cleavage of the caspase targets poly(ADP-ribosyl) polymerase and endogenous BAP31. In contrast, cytoplasmic membrane blebbing and fragmentation and apoptotic redistribution of actin were strongly inhibited, cell morphology was retained near normal, and the irreversible loss of cell growth potential following removal of the Fas stimulus was delayed. In its unmutated form, BAP31 is a preferred substrate for caspases 8 and 1 whose cleavage product generates a p20 fragment that remains integrated in the ER membrane (Fig. 3.8). When expressed ectopically, the p20 fragment is a potent inducer of cell death. These results argue that the cytosolic domain of BAP31 is important for regulating cytoplasmic apoptotic events associated with membrane fragmentation.

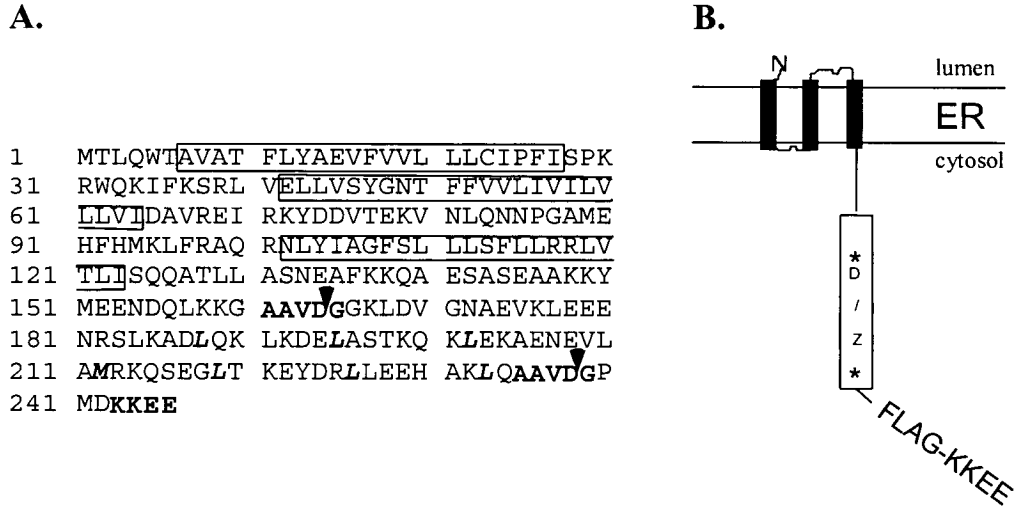


Figure 3.8. Polypeptide sequence and putative arrangement of Bap31 in the ER membrane. *A. Amino acid sequence of human BAP31(single-letter code; the sequence data is available from GenBank under accession X81817).* The three predicted transmembrane segments are boxed and the predicted caspase recognition sites, AAVD/G, are highlighted. 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. *B. Putative topology of BAP31 in the ER membrane.* 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. The FLAG construct used in this work has been inserted between amino acids 242 and 243.

To examine proteins that might be potentially associated with BAP31, BAP31-Flag was inserted and stably expressed in the human HepG2 cell line. The pre-apoptotic endoBAP31 complex was immunoprecipitated using the anti-Flag antibody and the immunopurified proteins were analyzed by SDS-PAGE. The immunocomplex was found to contain both the BAP31-Flag and the endogenous BAP31 proteins, migrating at apparent mobility of 33 kDa and 28 kDa, respectively, and two additional protein bands at apparent masses of 42 and 190 kDa (Nguyen et al., 2000). For sequence analysis, the BAP31 immunoprecipitation protocol was scaled up and the obtained immunocomplex was analyzed by preparative SDS-PAGE using a 10 %T separating gel (Fig. 3.9).

3.11.2 Peptide fragmentation mapping by LC-μESI-MS/MS

Specifically recruited proteins (at 190 kDa, 42 kDa and 28 kDa) were excised from the gel, destained, and in-gel digested for protein identification. Peptides obtained by the proteolytic in-gel digestion were analyzed by LC-μESI-MS/MS. Detailed analysis of the 28 kDa protein confirmed its identity as the endogenous BAP31 protein while the 42 and the 190 kDa band were identified as non-muscle γ-actin and myosin heavy chain non-muscle type B, respectively. Unambiguous identification was made difficult by the large number of described protein variants in the human database. Concomitantly, several good quality MS/MS spectra were not correlated to the database by SEQUEST, indicating the potential presence of post-translational modifications and/or additional protein variants. We therefore re-analyzed the obtained data with a smaller database, containing only

human actin or human myosin entries, and MS/MS spectrum that failed to be confidently identified in the first pass analysis were manually interpreted (Table 3.1).

45 of the initial 51 tandem mass spectrometric experiments obtained from the 190 kDa band LC-MS/MS analysis were selected for a second pass identification using a protein database filtered for the words "human" and "myosin" (144 entries). In total, 41 MS/MS spectra were assigned to myosin heavy chain non-muscle type B tryptic peptides, covering 33% of the total amino acid sequence (Fig. 3.10A). Two MS/MS spectra were found to contain peptides that deviated from the published amino acid sequence. One of them, at position 217-232, differed from the published amino acid sequence by a Ser227Ala mutation while 9 of the 14 amino acids of the second peptide at position 130-143 were exchanged. The mutated sequence was almost identical to a corresponding peptide in the skeletal muscle myosin heavy chain-2 sequence (MYHC-IIB; GeneBank accession number Q9Y623). In both cases, the predicted peptide and its mutated counterpart were present in an approximately equimolar amount, indicating either that two distinct myosin species (namely, myosin heavy chain non-muscle type B and skeletal muscle myosin heavy chain-2) were co-precipitated or that the non-muscle myosin expressed in the HepG2 cells was expressed in two (or several) allelic forms. The former hypothesis, however, is unlikely as these two myosin species share only about 40% identity. Therefore, several peptides specific for each myosin variant should have

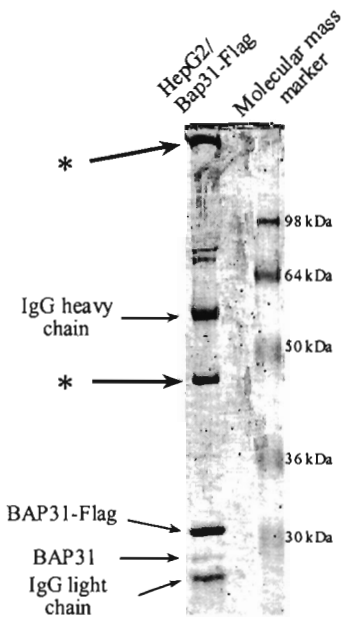


Figure 3.9. SDS-PAGE analysis of the immunoprecipitated pre-apoptotic BAP31-Flag complex in transfected HepG2 cells. Pre-apoptotic HepG2 cells, stably expressing the BAP31-Flag construct, were lysed and *BAP31* was immunoprecipitated with the anti-Flag M2 antibody. The immunocomplex was subjected to SDS-PAGE analysis and visualized by Coomassie blue staining. The two bands of interest, at apparent molecular masses of 42 and 175 kDa, are labeled with a star.

been identified during the LC-MS/MS analysis. Finally, four MS/MS spectra could not be unambiguously assigned to a given peptide sequence. All spectra were of medium quality and the absence of characteristical immonium ions specific for a terminal lysine or arginine might indicate that those peptides were not generated by a tryptic cleavage. As a result, they were not further analyzed.

Similarly, 39 spectra of the initial 42 tandem mass spectrometric experiments obtained from the 42 kDa band LC-MS/MS analysis were selected for a second pass identification using a protein database filtered for the words "human" and "actin" (280

Table 3.1. Overview of the tandem mass spectrometric analysis of myosin and actin by LC-MS/MS.

Analysis of the # of MS/MS spectra		database (# of entries)	identification
28 kDa band	15 MS/MS	human (67051 entries)	5 MS/MS: BAP31 human
			10 MS/MS: no identification
42 kDa band	42 MS/MS	human (67051 entries)	30 MS/MS β- or γ-actin
	39 MS/MS	"human" & "actin" (280 entries)	36 MS/MS γ-actin
			2 MS/MS: γ-actin variants
			1 MS/MS: no identification
180 kDa band	51 MS/MS	"human" (67051 entries)	35 MS/MS: myosin heavy chain non-muscle B
			16 MS/MS: no identification
	45 MS/MS	"human" & "myosin" (144 entries)	39 MS/MS: myosin heavy chain non-muscle B
			2 MS/MS: myosin variants
			4 MS/MS: no identification

A. Myosin heavy chain non-muscle type B (GeneBank accession P35580)

1. MAQRTGLEDPERYLFVDR**AVIYNPATQADWTAK**K**LVWIPSER**HGFEAASIKEERGDEVMV
61. ELAENGKKAMVNKDDIQKMNPPKFSKVEDMAELTCLNEASVLHNLKDRYYSGLIYTYSGL
121. FCVVINPYK**NLPIYSENIIEMYR**GKKRHEMPPHIYAISESAYRCMLQDREDQSILCTGES
WLPVYNPEVVAAYRGKKR
181. GAGKTENTKK**VIQYLAHVASSHK**GRKDHNIPGELER**QLLQANPILESFGNAK**TVKNDNSS
QLLQANPILEAFGNAK
241. RFGKFIR**INFDVTGYIVGANIETYLLEK**SRAVRQAKDERTFHIFYQLLSGAGEHLK**SDLL**
301. **LEGFNNYRFLSNGYIPIPGQQDK**DNFQETMEAMHIMGFSHEEILSMLKVVSSVLQFGNIS
361. FKKERNTDQASMPENTVAQKLCHLLGMNVMEFTRAILTPRIKVGRDYVQKAQTK**EQADFA**
421. **VEALAK**ATYERLFRWLVHRINKALDRTKRQGASFIGILDIAGFEIFELNSFEQLCINYTN
481. EK**LQQLFNHTMFILEQEEYQR**EGIEWNFIDFGLDLQPCIDLIERPANPPGVLALLDEECW
541. FPKATDKTFVEKLVQEQGSHSKFQKPRQLKDKADFCIIHYAGKVDYKADEWLMK**NMDPLN**
601. **DNVATLLHQSSDRFVAELWK**DVDR**IVGLDQVTGMTETAFGSAYK**TKKGMFRTVGQLYKES
661. LTKLMATLRNTNPNFVRCIIPNHEKRAGK**LDPHLVLDQLR**C*NGVLEGIRIC*RQGFPNR
719. IVFQEFR**QRYEILTPNAIPK**GFMDGKQACERMIR**ALELDPNLYR**IGQSKIFFRAGVLAHL
779. EEERDLKITDIIIFFQAVCRGCLARKAFAKKQQQLSALKVLQRNCAAYLK**LRHWQWWR**VF
839. TKVKPLLQVTRQEEELQAKDEELLKVKEKQTKVEGELEEMERKHQQLLEEKNILAEQLQA
899. ETELFAEAEEMRARLAAKKQELEEILHDLESRVEEEEERNQILQNEKKKMQAHIQDLEEQ
959. LDEEEGARQKLQLEKVTAEAKIKK**MEEEILLLEDQNSK**FIKEKKLMEDRIAECSSQLAEE
1019. EEKAKNLAKIR**NKQEVMISDLEER**LKKEEKTRQELEKAKR**KLDGETTDLQDQIAELQAQI**
1079. **DELK**LQLAKKEEELQGALARGDDETLHKNNALKVVRELQAQIAELQEDFESEKASRNKAE
1139. KQKR**DLSEELEALKTELEDTLDTTAAQQELR**TKREQEVAELKKALEEETKNHEAQIQDMR
1199. QR**HATALEELSEQLEQAK**RFKANLEKNKQGLETDNKELACEVKVLQQVKAESEHKRKKLD
1259. AQVQELHAKVSEGDRLRVELAEKASK**LQNELDNVSTLLEEAEKK**GIKFAK**DAASLESQLQ**
1319. **DTQELLQEETR**QKLNLSSRIRQLEEEKNSLQEQQEEEEEARKNLEKQVLALQSQLADTKK
1379. KVDDDLGTIESLEEAKKKLLKDAEALSQRLEEKALAYDKLEK**TKNRLQQELDDLTVDLDH**
1439. **QR**QVASNLEKKQK**KFDQLLAEEK**SISARYAEERDRAEAEAREKETKALSLARALEEALEA
1499. KEEFERQNKQLRADMEDLMSSKDDVGKNVHELEKSKR**ALEQQVEEMRTQLEELEDELQAT**
1559. **EDAK**LRLEVNMQAMKAQFERDLQTRDEQNEEKKRLLIKQVR**ELEAELEDER**KQRALAVAS
1619. KKKMEIDLKDLEAQIEAANKARDEVIKQLRKLQAQMKDYQRELEEARASRDEIFAQSKES
1679. EKKLK**SLEAEILQLQEELASSER**ARRHAEQERDELADEITNSASGKSALLDEKRRLEARI
1739. AQLEEELEEEQSNMELLNDRFRK**TTLQVDTLNAELAAER**SAAQKSDNARQQLERQNKELK
1799. AKLQELEGAVKSKFKATISALEAK**IGQLEEQLEQEAK**ERAAANKLVRRTEKKLKEIFMQV
1859. EDERRHADQYKEQMEKANARMKQLKRQLEEAEEEATRANASRRKLQRELDDATEANEGLS
1919. REVSTLKNRLRRGGPISFSSSRSGRRQLHLEGASLELSDDDTESKTSDVNETQPPQSE

B. Non-muscle γ-actin (GeneBank accession P02571)

1. **Ac-EEEIAALVIDNGSGMCK**AGFAGDDAPR**AVFPSIVGRPR**HQGVMVGMGQKDSYVGDEA
EEEIAALVVDNGSGMCK
58. QSK**RGILTLKYPIEH*GIVTNWDDMEK**IWHHTFYNELR**VAPEEHPVLLTEAPLNPK**ANRE
117. K**MTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILR**
177. LDLAGR**DLTDYLMK**ILTER**GYSFTTTAER**EIVRDIKEKLCYVALDFEQEMATAASSSSLE
237. K**SYELPDGQVITIGNER**FR**CPEALFQPSFLGMESCGIHETTFNSIMK**CDVDIRK**DLYANT**
297. **VLSGGTTMYPGIADR**MQK**EITALAPSTMK**IKIIAPPER**KYSVWIGGSILASLSTFQQMWI**
357. **SKQEYDESGPSIVHR**KCF

Figure 3.10. Detailed amino acid sequence analysis of (A) the myosin heavy chain non-muscle type B (GeneBank accession P35580) and of (B) non-muscle γ-actin (GeneBank accession P02571) by LC-μESI-MS/MS. All amino acids are in single-letter code. The peptides unequivocally identified by spectral matching of the tandem mass spectra with the sequence database by SEQUEST are underlined. *Panel (A),* the two potential alkylated cysteine residues (SH1/SH2 sites) at position 701 and 711, respectively, are marked with a star. Two peptides were found to deviate from the predicted sequence (at position 130-147 and position 217-232). *Panel (B)*, the methyl-histidine at position 72 is marked with a star. The two N-terminal peptide variants described in this work are indicated. See text for more details.

entries). In total, 38 MS/MS spectra could be assigned to γ-actin tryptic peptides, covering 69% of the total amino acid sequence (Fig. 3.10b). In particular, the presence of a methyl-histidine reported in the literature at position 72 was confirmed in our analysis. Of particular interest were five MS/MS spectra of good spectral quality that could not be initially matched to any specific actin sequence. Manual interpretation of the fragmentation patterns (Fig 3.11) indicated that all five analyzed species were derived from a heterogeneous N-terminal peptide. Panel (A) shows the tandem mass spectrum of the N-terminal peptide as reported in the sequence database: the N-terminal Met residue has been removed and the first glutamic acid residue has been acetylated. In addition, Cys16 was alkylated by an acrylamide monomer, a common experimental artifact when un-alkylated proteins are purified through a SDS-PAGE step (Chiari et al., 1992). A very rich fragmentation pattern was essential to confirm the putative peptide sequence in its entirety. Further, two of the five analyzed peptides were found to differ from the one

shown in panel (A) by an addition of 16 and 32 mass units, respectively. The corresponding fragmentation patterns confirmed the presence of a Met-sulfoxide (+16 Da) and a Met-sulfone (+32 Da), respectively, at position 15 (data not shown). In contrast, the two remaining peptides differed from the one in displayed in panel A by a difference of 56 and 40 mass units, respectively. The interpretation of their fragmentation patterns (as shown in panel B) points out to a free N-terminal glutamic acid residue (-42 Da for the missing acetyl group) and an Ile9Val exchange (-14 Da) while one of the peptide bears a Met-sulfoxide at position 15 (+ 16 Da; data not shown). Interestingly, the

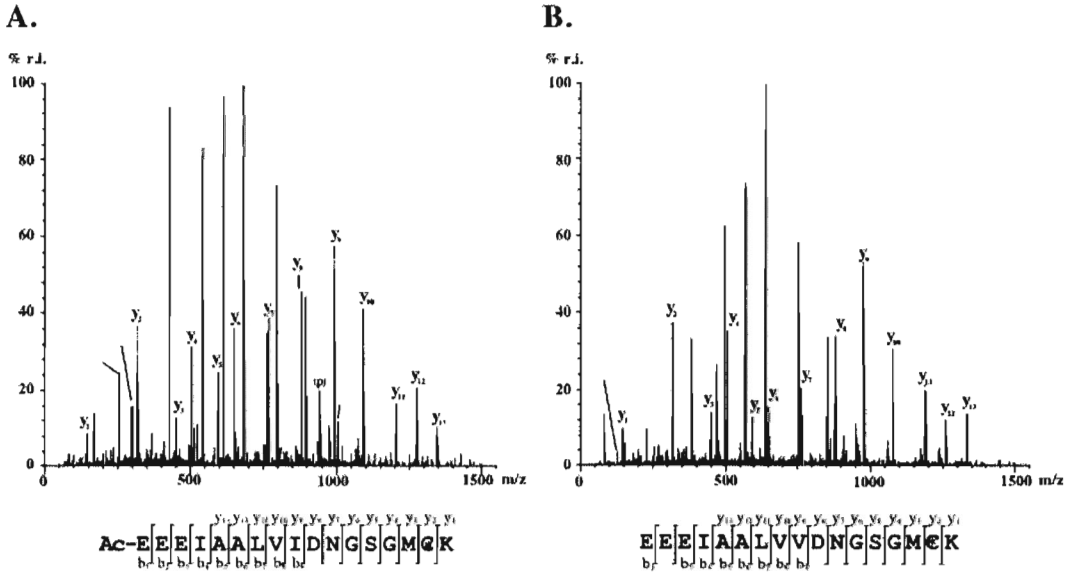


Fig. 3.11. Tandem mass spectrum analysis of the heterogeneous N-terminal peptides of γ actin. Panel (A), tandem mass spectrometric analysis of the acetylated N-terminal peptide with Ile at position 9. Panel (B), tandem mass spectrometric analysis of the de-acetylated N-terminal peptide with Val at position 9. The sequence coverage of each ion series is indicated in each panel in outline for the B-ion serie and in plain black for the Y-ion serie. See text for a more detailed discussion. Ac, acetyl; C*, S-cysteinyl-propionamide; (p), parent.

Met-sulfone derivative of this peptide was not detected during the LC-MS/MS analysis. Overall, only one MS/MS spectrum, of medium quality, could not be unambiguously assigned to an actin peptide sequence. The absence of characteristical immonium ions specific for a terminal lysine or arginine might indicate that this peptide was not generated by a tryptic cleavage. As a result, it was not further analyzed.

3.11.3 The pre-apoptotic BAP31 complex specifically recruits actomyosin

As documented above, the 42 and 190 kDa proteins that constitutively associates with BAP31 have been identified as γ-actin and non-muscle myosin II heavy chain B. Due to the abundance of those proteins in the cell, it was essential to demonstrate that these interactions were (a) specific to the presence of BAP31 in the immunoprecipitation complex and (b) that the association between BAP31 and the actomyosin complex was lost in the presence of an apoptotic signal. H1299 lung carcinoma cells were transiently transfected with vector, green fluorescent protein (GFP)-Flag, BAP31-Flag, or p20-Flag ("caspase-cleaved" BAP31; amino acids 1-164). The Flag-tagged proteins were precipitated from cell lysates 24 h following transfection using the anti-Flag M1 antibody and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using either anti-γ-actin or anti-Flag antibodies (Fig. 3.12A). All inserts were successfully expressed and an apparently equal amount of γ-actin was detected in all cell lysates. After immunoprecipitation with the anti-Flag antibody, however, γ-actin could only be detected in the BAP-31-immunocomplex. In particular, the immunocomplex containing p20-Flag, which contains the membrane-spanning domain of BAP31 but not its cytoplasmic tail,

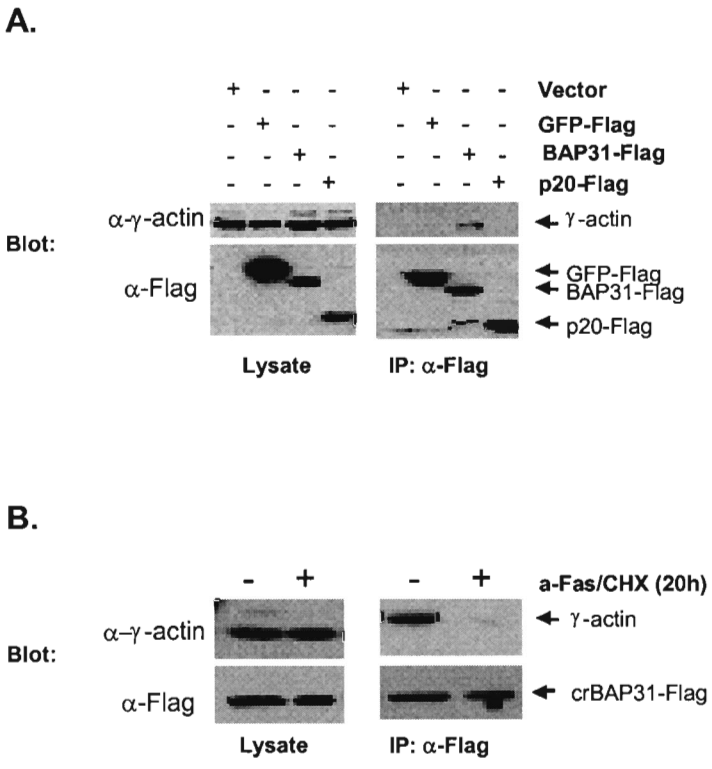


Figure 3.12. The pre-apoptotic BAP31 complex specifically recruits γ-actin. *Panel (A), Caspase-cleaved BAP31 (p20; amino acids 1 -164) does not interact with γ-actin.* H1299 lung carcinoma cells were transiently transfected with vector, GFP-Flag, BAP31-Flag, or p20-Flag. The Flag-tagged proteins were precipitated from cell lysates 24 h following transfection using anti-Flag H1 antibody (Upstate, Waltham, MA, USA) and immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-γ-actin or anti-Flag antibodies. *Panel (B), Loss of interaction between γ-actin and full length caspase-resistant crBAP31 following prolonged stimulation with Fas.* KB cells stably expressing crBAP31 were left untreated or treated with anti-FAS (250 ng/mL) and cyclohexamide (10 μg/mL) for 20 h to induce caspase activation and apoptosis. crBAP31 was immunoprecipitated from cell lysates using anti-Flag M1 antibody and immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-γ-Actin or anti-Flag antibodies.

was unable to recruit γ-actin, strongly indicating that the cytoplasmatic domain of BAP31 the association between BAP31 and γ-actin was the result of specific interactions.

is responsible for this interaction. A similar finding was observed in KB cells in which γ-actin association with endogenous BAP31 was rapidly lost following Fas stimulation, commensurate with cleavage of the BAP31-cytoplasmatic domain by caspases (data not shown). Furthermore, prolonged (20 h) Fas stimulation of KB cells stably expressing crBAP31-Flag (Nguyen et al., 2000), the caspase-resistant mutant of BAP31, also caused loss of these interactions even though the amounts of cellular crBAP31 and γ-actin did not apparently change (Fig 3.12B). These findings, coupled with the strong enrichment of the γ-isoform of actin recovered in the BAP31 immunoprecipitate, indicate that the association between BAP31 and γ-actin was the result of a specific interaction.

3.12 Discussion

We described in this paper the purification and the characterization of a BAP31 immunocomplex isolated from a pre-apoptotic human HepG2 cell lysate. The ubiquitously expressed 28 kDa integral ER membrane protein has been described as a potential regulator of cell death, by virtue of its association with procaspase-8L and the Bcl-2/Bcl-X_L protein family (Breckenridge et al., 2002; Ng et al., 1997). BAP31 has also been reported to associate with distal constituents of the ER secretory pathway, including IgD, cellubrevin and cystic fibrosis transmembrane conductance regulator, while recent evidence indicates that BAP31 is also involved in the transport of ER proteins to the Golgi (Annaert et al., 1997; Maatta et al., 2000).

Characterization of a BAP31 immunocomplex isolated from a pre-apoptotic human HepG2 cell lysate revealed the presence of BAP31-flag, the endogenous BAP31

protein (due to homo-oligomerization, (Ng and Shore, 1998), non-muscle myosin heavy chain type B, and non-muscle γ-actin, two components of the cytoskeleton actomyosin complex. A specific interaction of myosin with BAP31 is supported by the presence in the cytosolic coiled-coil region of BAP31 (from Leu122 to Ala236) of 4 segments of 4 heptads each similar to that observed in myosin heavy chain coils (Strehler et al., 1986). α-helical coiled-coil structures are known to mediate homo- and hetero-dimerization of proteins, and these sequences may facilitate the specific interactions between BAP31 and myosin heavy chain. Furthermore, the initial stage of cytoplasmic apoptosis – the release of extracellular matrix attachment and reorganization of focal adhesion, during which cell morphology is lost to adopt a round conformation (Mills et al., 1999) – is typically associated with changes in the organization of the cellular actomyosin complex, whereas actin rearranges into a peripheral ring in preparation for blebbing. In this context, the expression of the caspase-resistant BAP31 in KB epithelial cells subjected to apoptosis after Fas stimulation initially maintained a normal γ-actin distribution within the cell in contrast to the parental KB cells (Nguyen et al., 2000) whereas the ectopic expression of p20 BAP31 (lacking the 8 kDa C-terminal segment containing the myosin motif) in 293T cells causes dramatic membrane remodeling and is a potent inducer of cell death (Ng et al., 1997). It is noteworthy that neither procaspase-8L nor BCL-2 was detected in the pre-apoptotic BAP31-Flag immunocomplex, consistent with the requirements for a specific apoptotic stimulus, like that supplied by oncogenic E1A, to stimulate these interactions (Breckenridge et al., 2002).

An important aspect of this study was the use of tandem mass spectrometry to identify the protein bands co-purified with the immunoprecipitation of BAP31.

Identification of proteins separated by 1D- or 2D-SDS-PAGE is usually performed by peptide mass fingerprinting, wherein protein identification is obtained by the correlation of a collective of experimentally-measured masses with a computer-generated list of masses obtained from the *in silico* proteolytic cleavage of proteins in a database. While this method is usually successful if the protein of interest is present in the database, a number of experimentally determined masses typically will not match with the computer-generated list. One likely explanation (among others) is the presence of unexpected post-translational modifications or experimental artifacts, which cannot be easily accounted for if their mass increments from the unmodified peptides have not been solidly experimentally documented. In many instances, a definite answer might require a detailed sequence analysis, typically by N-terminal sequencing (using Edman degradation) or by tandem mass spectrometry. In the latter case, each analyzed peptide is fragmented into its constituent amino acid sequence and each resulting spectrum is individually correlated to the sequence database.

In this study, where a sufficient amount of starting material was available for a detailed analysis, we have attempted to match every obtained tandem mass spectrum to the myosin or actin protein sequences. As a result, we obtained a very high sequence coverage (33% for myosin and 69% for actin) that lead us to conclude that the analyzed SDS-PAGE bands, to the extent of the available sequence information, only contained the proteins of interest. In addition, we were able to determine a number of sequence variations that are likely to have arisen at the genetic level (by the presence of two or more allelic copies of the gene of interest) rather than by the presence of low-level amount of contaminating proteins in the immunocomplex. In particular, all observed

single amino acid exchanges (Ser227Ala in the myosin sequence; Ile9Val in the actin sequence) can be traced back to single nucleotide polymorphisms. The alternate peptide found in the myosin protein sequence (from Trp130 to Arg143) might have arisen from a homologous recombination between the skeletal and the non-muscle myosin genes, followed by a single nuclear polymorphism (Thr to Ala) at position 140. However, we could not characterize another peptide encoded by this particular exon to unambiguously support this hypothesis. Finally, we found an unexpected variability at the level of acetylation of the N-terminal actin peptide. The N-terminal peptide containing Ile at position 9 was completely N-terminal acetylated while its counterpart with a Val at position 9 always contained a free N-terminus. In contrast to many other acetylated proteins, removal of the initiator Met residue and acetylation of the glutamic acid residue is important in regulating the interaction between actin and myosin in the actomyosin complex (Abe et al., 2000; Polevoda and Sherman, 2000). The acetylation of the N-terminus removes a positive charge and increases the interaction between actin and myosin compared to its de-acetylated counterpart. However, it is noteworthy that none of these mutations has been described in the publicly-available genomic databases and that some of them might therefore be restricted to this lab-grown HepG2 cell line. In particular, the biological significance of the variable N-terminal acetylation of γ-actin will have to be investigated in additional biological systems.

3.13 Acknowledgments

The authors are grateful to J. Mortimer, Merck Frosst Center for Therapeutic Research, for the genomic analysis of actin and myosin.

Chapter 4

Caspase Cleavage Product of BAP31 induces Mitochondrial Fission through Endoplasmic Reticulum Calcium Signals, Enhancing Cytochrome c Release in Intact Cells

4.1 Rationale

Cells expressing crBAP31 selectively resisted two main features of apoptosis: 1) cytoplasmic morphological rearrangements including plasma membrane blebbing/integrity, cell shrinkage, nuclear condensation, and actin depolymerization; and 2) mitochondrial apoptotic transitions, including organelle clustering and fragmentation, cyt.c release, and loss of $\Delta\Psi_m$. It is possible that BAP31 cleavage has independent effects on these two apoptotic processes, or that BAP31 primarily regulates one of these features, which, in turn, promotes the onset of the other. For example, through its association with the actomyosin network, BAP31 might regulate cytoskeletal dynamics that are known to control cytoplasmic apoptosis (membrane blebbing, nuclear condensation etc) (Mills et al., 1999). BAP31's effects on the cytoskeleton might then indirectly alter mitochondrial dysfunction perhaps by effecting mitochondrial localization (De Vos et al., 1998), or the access of apoptotic factors to mitochondria. In contrast, BAP31 might directly affect apoptotic cross-talk between the ER and mitochondria, and mitochondrial release of apoptogenic factors might, in turn, regulate cytoplasmic apoptosis.

In chapter 4, I decided to examine the mechanism by which the p20 caspase cleavage product of BAP31 induces apoptosis. As discussed above, the phenotype conferred by crBAP31 might simply represent its ability to exert a DN influence on proapoptotic p20 and, therefore, determining the early cellular changes following p20 expression might yield clues into what processes are directly effected by BAP31 cleavage during physiological apoptosis.

4.2 Abstract

Stimulation of cell surface death receptors activates caspase-8, which targets a limited number of substrates including BAP31, an integral membrane protein of the endoplasmic reticulum (ER). We recently reported that a caspase-resistant BAP31 (crBAP31) mutant inhibited several features of Fas-induced apoptosis, including the release of cytochrome c (cyt.c) from mitochondria (Nguyen, M., D.G. Breckenridge, A. Ducret, and G.C. Shore. 2000. *Mol. Cell Biol.* 20:6731-6740), implicating ER-mitochondria crosstalk in this pathway. Here, we report that the p20 caspase cleavage fragment of BAP31 can direct proapoptotic signals between the ER and mitochondria. Adenoviral expression of p20 caused an early release of Ca^{2+} from the ER, concomitant uptake of Ca^{2+} into mitochondria, and mitochondrial recruitment of Drp1, a dynamin related protein that mediates scission of the outer mitochondrial membrane, resulting in dramatic fragmentation and fission of the mitochondrial network. Inhibition of Drp1 or ER-mitochondrial Ca^{2+} signaling prevented p20-induced fission of mitochondria. p20 strongly sensitized mitochondria to caspase-8-induced cyt.c release, whereas prolonged expression of p20 on its own ultimately induced caspase activation and apoptosis through the mitochondrial apoptosome stress pathway. Therefore, caspase-8 cleavage of BAP31 at the ER stimulates Ca^{2+}- dependent mitochondrial fission, enhancing the release of cyt.c in response to this initiator caspase.

4.3 Introduction

Mitochondria are key regulators of apoptosis that integrate diverse apoptotic stimuli into a core death pathway (Green and Reed, 1998). Mitochondrial control of apoptosis is

governed by the Bcl-2 family of proteins, which include antiapoptotic BCL-2 and BCL-xL and proapoptotic BAX and BAK; the balance between these opposing members is regulated by a third subgroup called the "BH3-only" proteins (Cory and Adams, 2002). Current models hold that BH3-only proteins invoke a mitochondrial phase of apoptosis by directing the insertion of BAX into mitochondria and inducing oligomerization of BAX and BAK in the outer mitochondrial membrane (OMM), causing an efflux of intermembrane space proteins, including cyt.c (Korsmeyer et al., 2000). Once in the cytosol, cyt.c complexes with Apaf-1 and procaspase-9 forming the apoptosome, a direct activator of downstream effector caspases -3 and -7 (Budihardjo et al., 1999). Activation of the TNF family of cell surface death receptors is coupled to the mitochondrial phase of apoptosis by the BH3-only protein BID. For example, binding of Fas to its ligand or agonistic antibody induces the recruitment and autoactivation of initiator procaspase-8 (Krammer, 2000). Caspase-8, in turn, cleaves BID generating tBID, which translocates from the cytosol to mitochondria and induces organelle dysfunction and cyt.c release (Li et al., 1998; Luo et al., 1998). BID plays an obligate role in transducing signals from death receptors to mitochondria in at least some cell types since hepatocytes from BID-/- mice do not release cyt.c in response to Fas, despite normal activation of caspase-8 (Yin et al., 1999). In some contexts caspase-8 can bypass mitochondria and directly cleave downstream caspases. In many cell types, however, the BID-dependent mitochondrial loop is required to amplify weak death receptor signals and relieve the inhibitory effect of IAP proteins on caspase activity (Deng et al., 2002; Fulda et al., 2002; Scaffidi et al., 1998; Yin et al., 1999).

While it is clear that Bcl-2 family members govern mitochondrial dysfunction it remains unclear at what point the functions of these proteins intercede with gross

alterations in mitochondrial morphology that occur during apoptosis. Normal mitochondrial morphology can vary dramatically between cell types, but in most cases mitochondria form long "worm-like" tubules that may (Rizzuto et al., 1998) or may not (Collins et al., 2002) make up lumenally interconnected networks. The distribution of mitochondria depends on interactions with microtubules whereas mitochondrial size and shape is the result of constant fusion and fission processes (Bereiter-Hahn and Voth, 1994). Little is known about the mechanism of mitochondrial fission and fusion except that it is regulated by a group of evolutionary conserved GTPases; fusion is dependent on Fzo/Mfn, whereas fission relies on a dynamin related protein, Drp1, which severs the OMM (Osteryoung, 2001; Shaw and Nunnari, 2002). During apoptosis mitochondria remodel inner membrane cristae (Scorrano et al., 2002), fragment into small punctiform organelles that sometimes cluster in the perinuclear region (Desagher and Martinou, 2000; Frank et al., 2001; Pinton et al., 2001), and eventually undergo matrix swelling leading to OMM rupture (Mootha et al., 2001; Petit et al., 1998). Recently, Youle and colleagues demonstrated that fragmentation of the mitochondrial network during apoptosis is caused by large-scale activation of Drp1-dependent mitochondrial fission, and that this event is requisite for the mitochondrial phase of apoptosis (Frank et al., 2001). How apoptotic signals converge on the fission machinery, however, is unclear.

In the current study we present evidence that caspase cleavage of BAP31 at the endoplasmic reticulum (ER) can trigger the onset of mitochondrial fission. BAP31 is a polytopic integral protein of the ER membrane that forms a large hetero-oligomeric complex with the related BAP29 protein and components of the actomyosin network (Adachi et al., 1996; Ng et al., 1997; Nguyen et al., 2000). Following activation of cell surface death receptors, human BAP31 is cleaved at two identical caspase recognition

sites in its cytosolic tail, generating a membrane embedded fragment, called p20, which induces apoptosis when expressed ectopically (Ng et al., 1997; Nguyen et al., 2000). Cleavage of BAP31 seems to be an important event in the Fas pathway since cells expressing a caspase-resistant BAP31 (crBAP31) mutant retain a near normal morphology following stimulation and resist apoptotic membrane blebbing/fragmentation, disruption of the actin network, and irreversible loss of cell growth potential following removal of the Fas stimulus (Nguyen et al., 2000). In addition, crBAP31 prevents mitochondrial remodeling and the release of cyt.c in the face of activated caspases, suggesting that events at the ER can modulate mitochondrial dysfunction in intact cells (Nguyen et al., 2000). To better understand this communication between ER and mitochondria, and how BAP31 contributes to Fas signaling in general, we investigated the role of p20 in apoptotic progression. We find that p20 stimulates ER Ca^{2+} release, resulting in the activation of Drp1-dependent fission of mitochondria, which ultimately sensitizes this organelle to caspase-8-induced cyt.c release.

4.4 Materials and Methods

Antibodies, plasmids and reagents - The following antibodies were employed in this study: Chicken anti-human BAP31 (Ng et al., 1997) rabbit polyclonal antibodies raised against the recombinant human BAP29, human TOM-20 (Goping et al., 1995), γ-actin (gift from P. Braun, McGill University, Montreal, PQ), and human BAX aa 1-21 (Upstate Biotechnologies); Rabbit polyclonal antibody raised against the p15 caspase cleavage

product of BID and purified by affinity selection; and mouse monoclonal antibodies to pigeon cytochrome c (Pharmingen), chicken α-tubulin (clone DM1A, Sigma-Aldrich), rodent Drp1 (BD Biosciences), and hemagluttin (HA) (Clone16B12, BABCO). Goat anti-calreticulin was provided by M. Michalak (University of Alberta, Edmonton, AB). Anti-human Fas activating antibody (Clone CH-11) was from Upstate Biotechnologies. Standard PCR techniques were used to generate cDNA encoding p20 (aa 1-164 of human BAP31) with a c-terminal Hemagluttin (HA) tag, which was cloned in to pcDNA3. Plasmids encoding Drp1 and $Drp1_{K38E}$ fused to CFP at the N-terminus were generous gifts from H. McBride (Ottawa Heart institute, Ottawa, ON). Carbobenzoxy-valvy-alanyl-aspartyl-methyl ester-fluormethyl ketone (zVAD-fmk) was purchased from Enzyme System Products. Fura2-AM, Rhod2-AM and BAPTA-AM were from Molecular Probes and Ru360 was from Calbiochem. All other chemicals were purchased from Sigma-Aldrich, unless otherwise noted.

Cell culture, virus infection, and transfection - KB epithelial cells and H1299 lung carcinoma cells were maintained in MEM-α supplemented with 10% FBS and 100 U/ml streptomycin and penicillin. Rat1 Fibroblast, CHO, and Hela cells were grown in DMEM supplemented as above. KB cells stably expressing HA-Bcl-2 and HA-Bcl-X_L have been described (Nguyen et al., 1998; Ruffolo et al., 2000). H1299 b5-Bcl-2 cells were created by transfecting H1299 cells with pcDNA3 vector encoding human HA-Bcl-2 with amino acids 215-239 swapped with the TM sequence of human cytochrome B5 (amino acids 107-134) and selecting for resistance to geneticin. *Bap31*-null, and *Bap29*-null mouse embryonic stem cells were maintained as described (Breckenridge et al., 2002).

For the construction of Adp20, AdMF_{pk}3FLICE, and AdRTA, cDNAs encoding p20-HA, MF_{pk}3FLICE (Muzio et al., 1998) and the reverse tet transactivating protein were subcloned into a variant of pCA14 containing the T-REx promoter (Invitrogen), which functioned as a shuttle to produce the adenoviral vectors as described (Bett et al., 1994; Mathai et al., 2002). All adenoviral infections were conducted at a multiplicity of infection (MOI) of 100 pfu/cell as described (Ng et al., 1997), except for in Fig. 4.3 where the viruses were mixed to generate a total MOI of 100 pfu/cell. Lipofectamine Plus (Invitrogen) was used for all transfections using the manufacturer's protocols. In experiments where Adp20 infection followed transfection of CFP-Drp1 or CFP-$Drp1_{K38E}$, the transfection medium was removed 3 h post transfection and medium containing serum and Adp20 virus was added back.

Apoptotic assays - DEVDase activity was measured from 25 μg of cell lysate according to the manufacturer's protocol (Upstate Biotechnology). For statistical analysis of mitochondrial fission and cyt.c release, cells were stained for TOM20 or cyt.c and the distribution of cyt.c and the morphology of mitochondria were analyzed by conventional immunofluorescence microscopy. In all cases at least 5 independent experiments were conducted, where 3 counts of 150 randomly selected cells was done per experiment. In Fig. 4.7B, only cells showing CFP expression were assessed for cyt.c release. Biochemical isolation of the heavy membrane fraction enriched in mitochondria or post-mitochondrial supernatant for measurement of cyt.c release and Drp1 recruitment was done as described (Ruffolo et al., 2000). In Fig. 4.3, the intensity of each cyt.c and tBID western blot signal was quantified using ImageQuant™ (Amersham) software and compared to the intensity of a loading control signal in the same lane (actin or TOM20,

respectively), following the subtraction of background. The relative values were expressed as arbitrary units.

Fluorescence microscopy - Cells were typically seeded at 50% confluency on glass coverslips and mock infected or infected with Adp20, always in the presence of zVAD-fmk to prevent apoptosis and cell detachment. At the indicated times post-infection, cells were washed in PBS then fixed in para-formaldehyde solution (4% para-formaldehyde, 23 mM NaH_2PO_4, 77 mM Na_2HPO_4, pH 7.3). Cells were briefly permeabilized in PBS/0.2% triton X-100, then blocked in blocking solution (PBS containing 10% FCS and 0.1% triton X-100). Primary and secondary antibody incubations were done in blocking solution for 1h at room temperature using the indicated antibodies and goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody coupled to Alexa 488 (Green) or Alexa 594 (red) (Molecular Probes). Cells were visualized by confocal microscopy or by conventional fluorescence microscopy on a TE-FM Epi-fl inverted microscope.

Measurement of ER Ca^{2+} content - The ER Ca^{2+} store was measured as the sudden increase in $[Ca^{2+}]_c$ upon addition of TG. $[Ca^{2+}]_c$ was measured by the cell permeable fluorescent indicator Fura2-AM. Briefly, $2X10^6$ cells were washed in Ca^{2+}-free buffer (20 mM Hepes pH 7.4, 143 mM NaCl, 6 mM KCL, 1mM $MgSO_4$, 0.1% glucose, 250 μM sulfinpyrazone), then loaded with Fura2-AM (3mM) for 30 min at 37°C in Ca^{2+}-free buffer containing 0.02% pluronic acid and 0.1% BSA. After a final wash, cells were resuspended in Ca^{2+}-free buffer and $[Ca^{2+}]_c$ was measured as 340/380 nm excitation wavelength ratio at 510 nm wavelength emission (340/380 ratio) in a LS 50B Perkin Elmer Luminescence spectrophotometer. The ER calcium content was measured as the

difference between the baseline 340/380 ratio before TG addition and the peak 340/380 ratio after TG addition. This value was arbitrarily set at 100% for untreated cells.

Measurement of $[Ca^{2+}]_m$ - $5X10^5$ cells were collected, washed once in PBS then resuspended in 1 ml Earl's balanced salt solution and loaded with Rhod2-AM (2 μM) in the presence of 0.02% pluronic acid for 20 min at room temperature. Cells were washed twice in the same buffer and Rhod2 fluorescence (F) measured as above at 550/580 excitation/emission wavelengths. Minimum and maximum fluorescence values (F_{max} and F_{min}, respectively) were then obtained upon the sequential addition of EGTA and saturating amounts of $CaCl_2$ in the presence of detergent. $[Ca^{2+}]_m$ was determined by the equation $[Ca^{2+}] = Kd\ (F - F_{min})/(F_{max} - F)$ where Kd is the dissociation constant of Rhod2. Rhod2 was judged to be localized to mitochondria based on analysis by immunofluorescence microscopy and by the fact that fluorescence was reduced to basal levels upon the addition of the mitochondrial uncoupler, CCCP.

4.5 Results

4.5.1 Caspase cleavage of BAP31 during Fas-mediated apoptosis generates p20.

BAP31 and its cellular homolog and heterodimerizing partner, BAP29 (Adachi et al., 1996), are structurally conserved proteins sharing identical topology in the ER membrane and 47% sequence identity in man. Both proteins initiate with the N-terminus facing the lumen, followed by three transmembrane (TM) regions and a cytosolic tail containing a long coiled coil domain ending in a canonical KKXX ER retrieval sequence (Fig. 4.1A). Human BAP31 contains two identical caspase cleavage sites (AAVD.G) at

D164 and D238 that are preferentially cleaved by caspase-8 (Ng et al., 1997). Figure 4.1B shows that in human KB cells stimulated with agonistic anti-Fas antibody BAP31 was cleaved generating the p27 and p20 membrane embedded fragments. Only the former cleavage site is conserved in mouse Bap31, suggesting that cleavage at D164 is critical. The two caspase cleavage sequences are not conserved in BAP29, which remained structurally intact during apoptosis (Fig 4.1B).

We previously observed that the p20 caspase cleavage fragment of BAP31 is cytotoxic when expressed ectopically (Ng et al., 1997), indicating that caspase cleavage of BAP31 might generate a proapoptotic gain of function. To study the mechanism of action of p20, we created an adenoviral vector (Adp20) expressing this fragment (aa 1-164 of human BAP31) with a C-terminal Hemagluttin (HA) tag. Endogenous p20 protein generated during Fas-mediated apoptosis was associated with microsomes and remained resistant to alkali extraction (pH 11.5), indicative of a membrane-integrated protein (data not shown). Immunofluorescence microscopic analysis of Adp20 infected human H1299 cells revealed that exogenous p20 strongly colocalized with endogenous calreticulin, a resident ER lumen protein, but p20 did not colocalize with TOM20, a marker of the OMM (Fig 4.1C). Therefore, caspase cleavage of BAP31 generates a proapoptotic p20 fragment that remains at the ER.

4.5.2 Prolonged expression of p20 induces apoptosis.

Expression of p20 was observed by 10 h following infection of KB cells with Adp20 and remained stable for over 50 h (Fig. 4.2A). 30-40 h post-infection, Adp20 induced the activation of caspases, measured by the hydrolysis of the caspase substrate

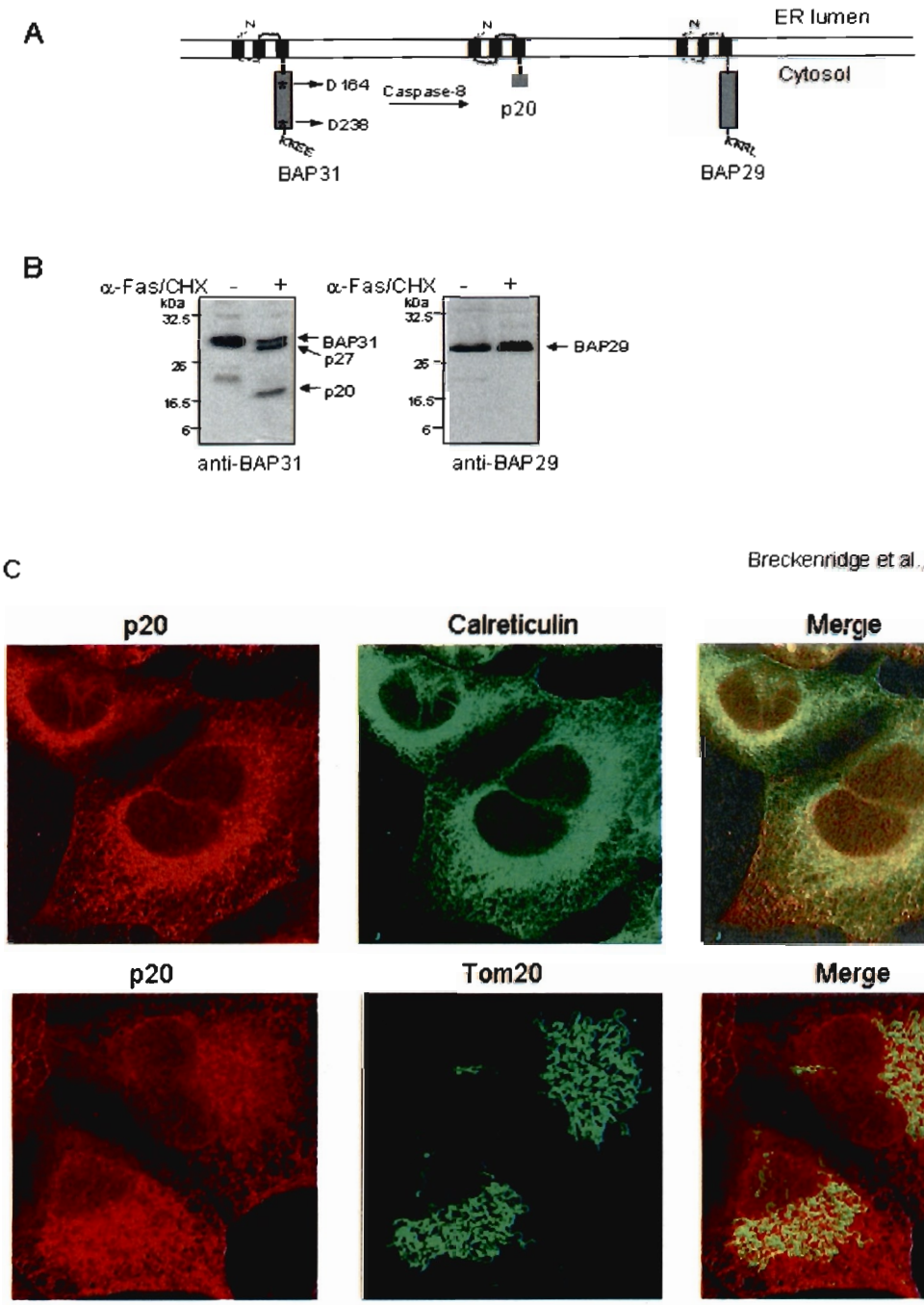


Figure 4.1. BAP31, but not BAP29, is cleaved during Fas-mediated apoptosis. (A) Schematic representation of human BAP31, the p20 caspase cleavage product, and BAP29 in the ER membrane. Both BAP31 and BAP29 contain three transmembrane domains, a cytosolic tail containing a coiled coil domain (boxed region), and terminate with a canonical KKXX ER retrieval sequence. The caspase-8 recognition sites in BAP31 are shown. (B) KB cells were untreated or stimulated with 500 ng/ml anti-Fas activating antibody (CH11) and 10 μg/ml cyclohexamide (CHX) for 7 h and cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-BAP31 (left) or anti-BAP29 (right) polyclonal antibodies. The positions of BAP31, its p27 and p20 cleavage products, and BAP29 are indicated. (C) Adenoviral expressed p20-HA (Adp20) localizes to the ER. H1299 cells were infected with Adp20 for 20 h then fixed and double stained with anti-HA and anti-calreticulin antibodies or anti-HA and anti-TOM20 antibodies.

DEVD-amc and by processing of procaspase-3, in many cell types including KB, H1299, Hela and Rat1 cells (Fig 4.2B, and data not shown). The mechanism of this caspase activation seemed to occur via the classical mitochondrial apoptosome stress pathway. For example, p20 expression resulted in the insertion of BAX into the OMM, homo-oligomerization of BAK, and release of cyt.c from mitochondria in the presence of the pan-caspase inhibitor, zVAD-fmk (Fig. 4.2C and unpublished data). In contrast, p20-induced caspase activation was abrogated in *APAF-1*-null cells (supplemental Figure 4.1). Control adenovectors expressing either LacZ or the reverse tet transactivating protein (RTA) did not cause any of the aforementioned apoptotic changes (data not shown). Inhibition of caspases using zVAD-fmk, or overexpression of BCL-2 or BCL-xL, blocked downstream morphological features of apoptosis including loss of plasma membrane integrity as assessed by trypan blue uptake (Fig 4.2D). In the absence of these inhibitors cells showed typical signs of apoptosis, including nuclear condensation and fragmentation, membrane blebbing and cell surface exposure of phosphatidlyserine (data not shown).

p20 strongly heterodimerizes with full length BAP31 (Nguyen et al., 2000) and, therefore, might cause apoptosis by exerting a dominant negative influence on endogenous BAP31 or BAP29. As shown in Figure 4.2E, however, cell death was observed in Bap31- or Bap29, 31-double deleted mouse cells (Breckenridge et al., 2002) infected with Adp20, demonstrating that p20 has an intrinsic proapoptotic activity at the ER that is separate from the functions of BAP31 and BAP29. Moreover, VSV-G export from the ER occurred at 22h post-Adp20 infection, suggesting that p20 does not exert a gross influence on ER-Golgi trafficking at this early time (supplemental Figure 4.2).

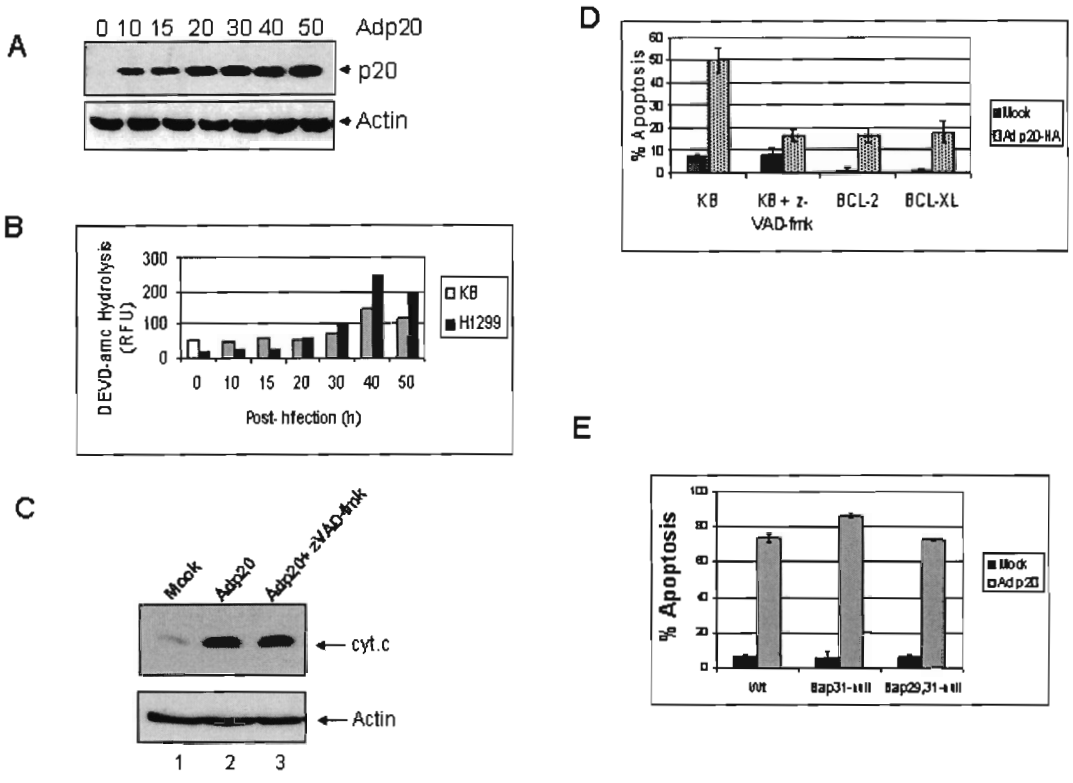


Figure 4.2. Prolonged expression of p20 induces mitochondrial apoptosis. (A) Expression of p20 in KB cells. Cells were infected with Adp20 and cell lysates collected and analyzed by immunoblotting at the times indicated post-infection. (B) KB and H1299 cells were infected with Adp20 and effector caspase (DEVDase) activity was measured at the indicated times post-infection by the ability of cell lysates to hydrolyze the fluorogenic caspase substrate DEVD-amc. Shown is a representative experiment. (C) KB cells were mock infected or infected with Adp20 for 35-40 h in the absence or presence of 50 μM zVAD-fmk and equivalent amounts of post-mitochondrial supernatants were analyzed for the presence of cyt.c by SDS-PAGE and immunoblotting. The membrane was reprobed with anti-actin antibody to confirm equal loading. (D) Parental KB cells, or KB cells stably over-expressing BCL-2 or BCL-xLwere mock infected or infected with Adp20, in the absence or presence of 50 μM zVAD-fmk, and at 45 h post infection cell death was assessed by trypan blue staining. Shown is mean $\pm$ SD of 3 independent experiments. (E) Wt, *Bap31*-null and *Bap29, 31*-null mouse ES cells were treated and analyzed as in (D).

Collectively, these results indicate that p20 can activate mitochondrial apoptosis. It is noteworthy, however, that this pathway did not culminate until at least 30-40 h post-infection (Fig. 4.2), whereas ectopic tBID induces cyt.c release within several hours of its expression (Li et al., 1998). A more relevant function for p20 in physiological cell death, therefore, might relate to an early sensitization of mitochondria to a co-stimulus.

4.5.3 p20 sensitizes mitochondria to caspase-8-induced cyt.c release.

Given that BAP31 is a caspase-8 substrate, p20 might cooperate with other products generated by caspase-8, such as tBID, to enhance mitochondrial dysfunction. According to this model, immediately after its expression p20 should activate a signal that is slow to induce cyt.c release on its own, but able to synergize with other apoptotic signals during Fas-mediated apoptosis. Therefore, we investigated whether p20 could enhance caspase-8-driven cyt.c release. Death receptor-dependent caspase-8 activation was mimicked by infecting H1299 cells with adenovector expressing triplicate copies of F_{pk} (a mutant of FKBP) fused to the catalytic subunits of caspase-8 ($AdMF_{pk}3FLICE$) (Muzio et al., 1998). Following its expression in cells, oligomerization and autoactivation of the caspase-8 fusion protein was induced with the F_{pk}-dimerizing compound, FK1012Z. This approach has the benefit of delivering synchronized caspase-8 signals to cells without stimulating caspase-8-independent pathways activated by death receptors (Schulze-Osthoff et al., 1998; Wang et al., 2001). In figure 4.3, H1299 cells were co-infected with AdRTA (control adenovector) and $AdMF_{pk}3FLICE$, or with Adp20 and $AdMF_{pk}3FLICE$. 16h post-infection, a time when Adp20 alone did not induce cyt.c release or caspase activation (Figs 4.2 and 4.3), the cells were exposed to a short treatment (45 or 90 min) with FK1012Z or vehicle (DMSO) alone, and the mitochondrial and post-mitochondrial fractions isolated. Compared to caspase-8 activation in the presence of the control protein RTA, caspase-8 activation in the presence of ectopic p20 strongly induced release of cyt.c to the cytosol (Fig 4.3A), but it did not affect the amount of caspase-8 generated tBID that was recovered in the mitochondrial fraction (Fig 4.3B). In all cases, equivalent amounts of $MF_{pk}3FLICE$ were produced (not shown). These

results suggest, therefore, that p20-mediated signals from the ER might cooperate with other caspase-8 generated signals to increase cyt.c release from mitochondria.

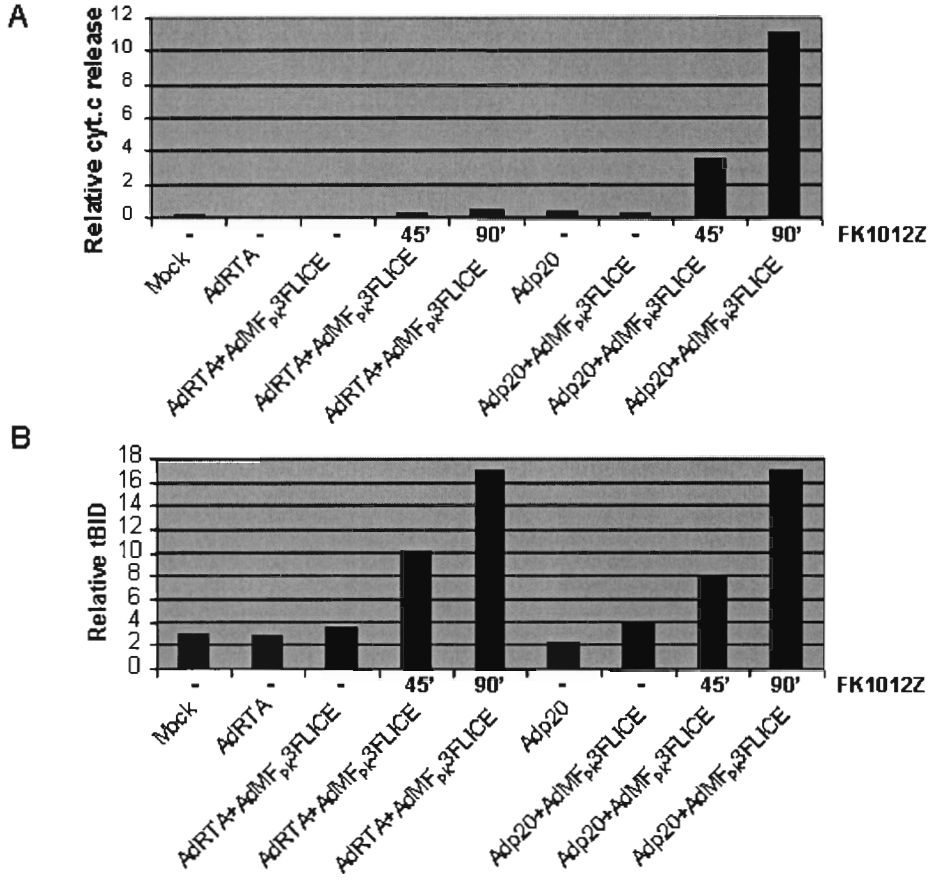


Figure 4.3. p20 sensitizes mitochondria to caspase-8-induced cyt.c release. H1299 cells were mock infected, or co-infected with AdRTA (control) and $AdMF_{pk}3FLICE$ or Adp20 and $AdMF_{pk}3FLICE$. 16 h post-infection, FK1012Z or vehicle alone (DMSO) were added for 45 or 90 min and the amount of cyt.c in the post-mitochondrial supernatant and tBID in the mitochondrial fraction were assessed by SDS-PAGE and western blot. The intensity of the cyt.c and tBID signals, relative to loading controls, was determined using ImageQuant™ software (Amersham) and is expressed in arbitrary units. Shown is a representative of three independent experiments.

4.5.4 p20 mediates its effect through an early release of Ca^{2+} from the ER.

We next sought to identify the early ER signaling events following p20 expression. Release of Ca^{2+} from the ER occurs as an early event during many forms of

apoptosis, including the Fas pathway, and Ca^{2+} has been implicated as a second messenger between ER and mitochondria during apoptosis (Breckenridge and Shore, 2002; Hajnoczky et al., 2000). We tested whether p20 expression altered ER Ca^{2+} homeostasis by loading Adp20 infected cells with the Ca^{2+} sensitive fluorescent indicator Fura-2AM and measuring the increase in cytosolic Ca^{2+} that results from thapsigargin- (TG) induced depletion of ER stores. TG invokes an immediate emptying of ER Ca^{2+} stores to the cytosol by irreversibly inhibiting SERCA pumps that normally maintain the concentration of ER Ca^{2+} ($[Ca^{2+}]_{ER}$) several orders of magnitude above that of the cytosol ($[Ca^{2+}]_c$). Figure 4.4A reveals that expression of p20 in H1299 cells in the presence of zVAD-fmk caused an early, time-dependent decrease in ER Ca^{2+} stores. The kinetics of ER Ca^{2+} release was concomitant with an increase in the concentration of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$), measured by Rhod2 fluorescence. These changes in ER and mitochondrial Ca^{2+} levels could be measured as early as 12-14 h post-infection (i.e. 2-4 h after p20 protein appears, Fig 4.2A) making them the earliest events we observed in the p20 pathway.

To determine whether the release of ER Ca^{2+} affected early responses of mitochondria to p20, we examined the consequence of inhibiting Ca^{2+} signaling between the ER and mitochondria. We began by adopting two experimental conditions that reduce the amount of Ca^{2+} that could be released from the ER by p20 (Pinton et al., 2001). In the first case, H1299 cells were incubated with a low concentration of TG (50 nM). Addition of TG resulted in an immediate emptying of ER Ca^{2+} stores, which remained depleted for over 24 h (supplemental Figure 4.3A). In a second approach we took advantage of the ability of BCL-2 to lower the $[Ca^{2+}]_{ER}$ by increasing the passive leak of Ca^{2+} from the organelle (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000). However, to minimize the

antiapoptotic activity of BCL-2 at the mitochondria, we created H1229 cells stably overexpressing BCL-2 selectively targeted to the ER with the membrane insertion sequence of cytochrome b5 (H1299 b5-BCL-2) (Zhu et al., 1996). H1299 b5-BCL-2 cells had an approximately 40% reduction in resting ER Ca^{2+} levels, resulting in a substantial decrease in the total amount of Ca^{2+} released in response to Adp20 (supplemental Figure 4.3B). We also tested the effect two pharmacological agents: BAPTA-AM, a cytosolic Ca^{2+} chelator that can inhibit Ca^{2+} transmission between the ER and mitochondria (Byrne et al., 1999; Sharma et al., 2000), and Ru360, an inhibitor of mitochondrial Ca^{2+} uptake (Matlib et al., 1998).

H1299 cells treated with Adp20+zVAD-fmk for 24 h and stained with cyt.c displayed dramatically fragmented mitochondria compared to mock-infected cells (Fig 4.4B). Remarkably, in cells pretreated with TG or expressing b5-BCL-2, the mitochondrial network remained intact and highly interconnected with no signs of mitochondrial fragmentation (Fig. 4.4B, data not shown). Quantification of the two mitochondrial phenotypes revealed that TG and b5-BCL-2 reduced the number of cells showing signs of mitochondrial fragmentation from 52% to 10% and 13%, respectively (Fig. 4.4C). Pretreatment of cells with BAPTA or Ru360 also reduced the number of cells manifesting fragmented mitochondria in response to p20 (Fig. 4.4C). The expression of p20 was not affected by any of the treatments (data not shown). TG, b5-BCL-2, BAPTA and Ru360 also inhibited p20-induced release of cyt.c from mitochondria, which occurred subsequent to fragmentation (data not shown, see below). Inhibition of Ca^{2+} transport between the ER and mitochondria, therefore, inhibits the effect of p20 on mitochondrial morphology and redistribution of cyt.c.

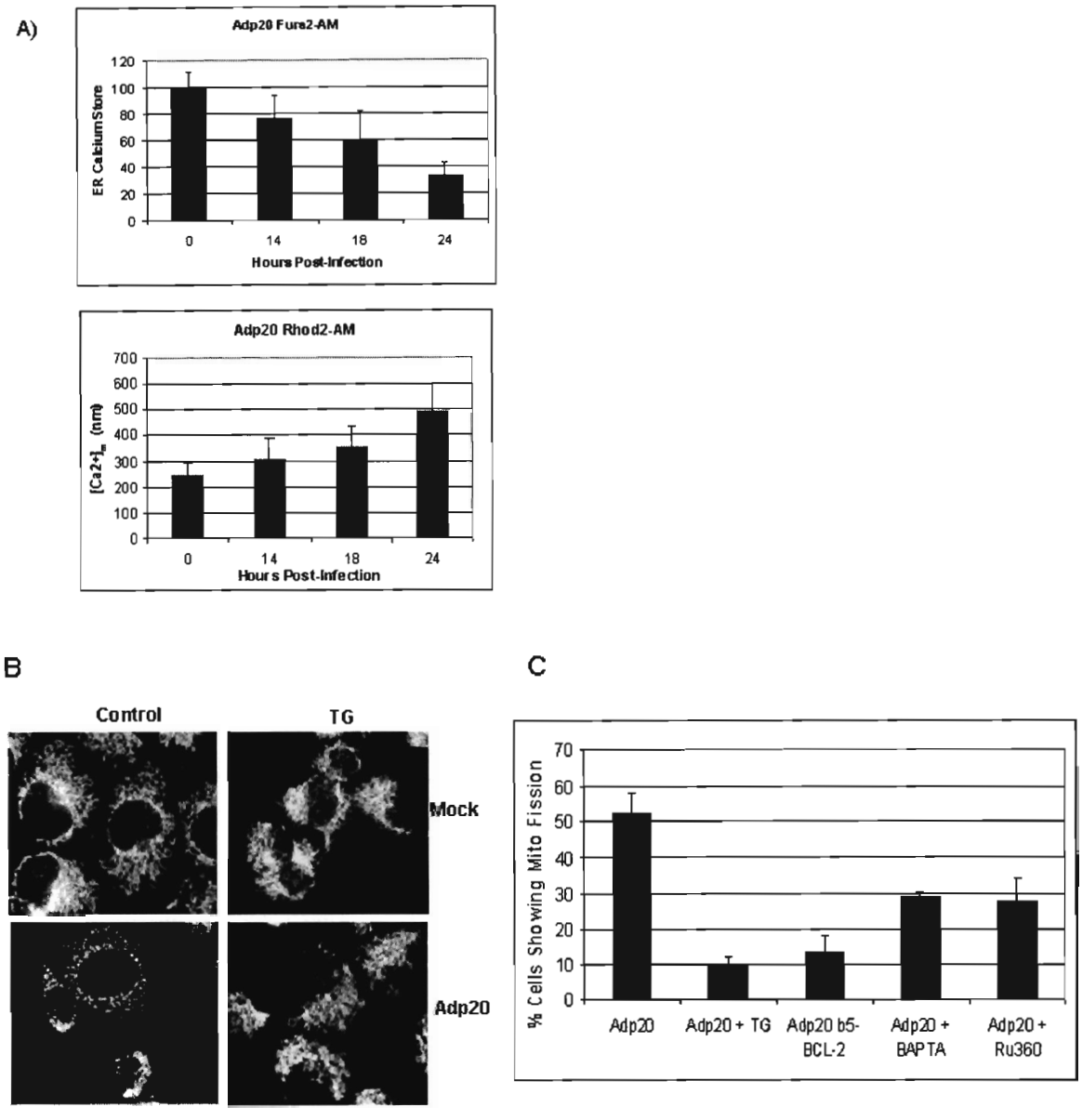


Figure 4.4. p20-induced mitochondrial fragmentation is mediated by an ER-mitochondria Ca^{2+} signal. (A) Top, p20 induces a time dependent release of Ca^{2+} from the ER. H1299 cells were infected with Adp20 in the presence of zVAD-fmk (50 μM) and at the indicated times post-infection cells were loaded with Fura-2-AM in Ca^{2+} free buffer and ER calcium stores were measured as the sudden difference in Fura-2 fluorescence recorded after the addition of thapsigargin (TG) (see Materials and Methods). Shown is the mean and SD of 5 independent experiments. Bottom, elevated $[Ca^{2+}]_m$ following Adp20 infection. Hela cells were treated as in (A) except cells were loaded with Rhod2-AM and the $[Ca^{2+}]_m$ was estimated as described in the Materials and Methods. (B) p20 induces dramatic fragmentation of mitochondria, which is inhibited by predepletion of ER Ca^{2+} stores with TG. H1299 cells were infected with Adp20 + zVAD-fmk (50 μM) in the absence or presence of 50 nm TG for 24 h and mitochondria were visualized by anti-cyt.c staining. Representative images are shown. (C) Reducing ER Ca^{2+} stores, chelating cytosolic Ca^{2+}, or preventing mitochondrial Ca^{2+} uptake inhibits p20-induced fragmentation of mitochondria. As in (B) but H1229 cells, or H1299 cells pretreated with 50 nm TG, 2 μM BAPTA-AM, or 20 μM Ru360, or H1299 b5-BCL-2 cells were infected with Adp20 + zVAD-fmk for 24 h and the number of cells showing signs of mitochondrial fragmentation was quantified. Shown is the mean $\pm$ SD of 5 independent experiments.

4.5.5 p20- induced fragmentation of the mitochondrial network.

The observation that p20 caused mitochondrial fragmentation was extended in Figure 4.5. p20 induced an early fragmentation of the mitochondrial network into small punctiform organelles in all cell types tested, including H1299, Rat1, and Hela cells (Figs 4.4 and 4.5, and data not shown). The gross morphological changes in the mitochondrial network could be observed by 15-16 h post Adp20 infection (i.e. 2-3 h following the onset of Ca^{2+} release), a time when p20 sensitized mitochondria to caspase-8-induced cyt.c release (Fig. 4.3). Induction of mitochondrial fragmentation by p20 occurred in the absence of zVAD-fmk sensitive caspase-activation and cell shrinkage or disruption of microtubules. For example, Figure 4.5A shows that Rat1 fibroblasts expressing p20+zVAD-fmk for 20 h and co-stained with anti-tubulin and anti-TOM20 antibodies displayed a normal microtubule distribution despite having fragmented mitochondria. The punctiform mitochondria could be observed in living cells stained with MitoTracker Red (unpublished data), indicating that fragmented mitochondria maintain membrane potential and were not an artifact of fixation. Co-staining of Rat1 fibroblasts expressing p20+zVAD-fmk with antibody to cyt.c and antibody selective for the active conformation of BAX (Desagher et al., 1999) revealed that the transition of mitochondria into punctiform organelles preceded cyt.c release and activation of BAX. As exemplified in Figure 4.5B, most cells expressing p20+zVAD-fmk for 25 h displayed fragmented mitochondria but showed no signs of cyt.c release or BAX immunoreactivity. BAX immunoreactivity could only be observed in apoptotic cells that had released cyt.c from the mitochondria, and all cells that had undergone cyt.c release stained positive for BAX. These results suggest that p20 induces early fragmentation of mitochondria, which precedes BAX activation and cyt.c release. Given that disintegration of the mitochondrial

network has been demonstrated to contribute to apoptotic progression (Desagher and Martinou, 2000; Frank et al., 2001) but that BAX/BAK activation and cyt.c release are normally stimulated by BH3-only molecules (Korsmeyer et al., 2000), it is likely that p20 mediates its sensitizing effect by inducing early fragmentation of mitochondria.

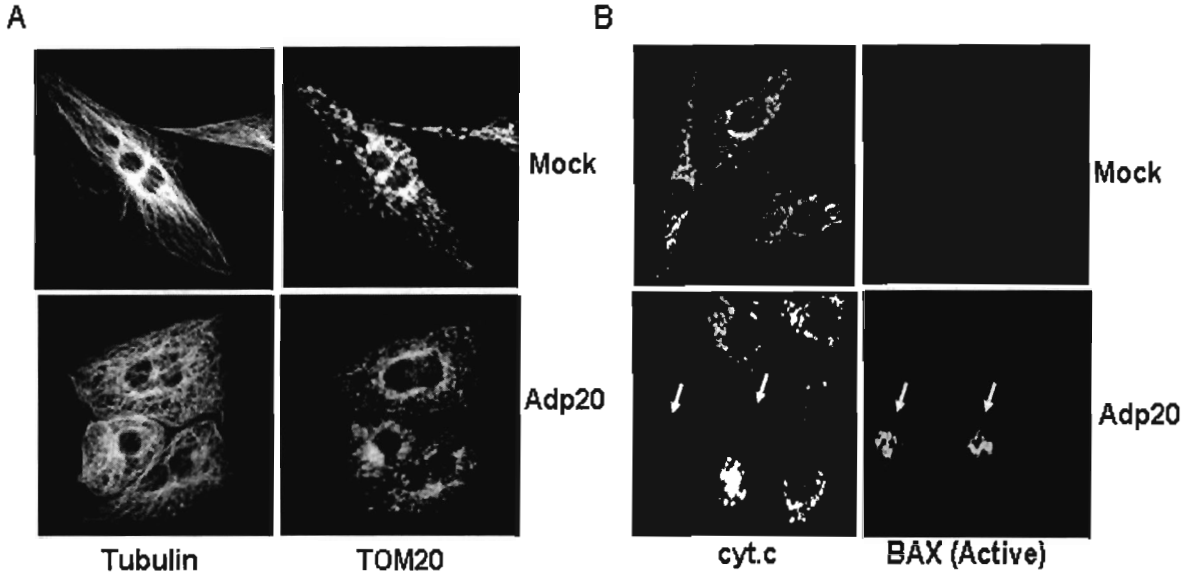


Figure 4.5. p20 induces fragmentation of the mitochondrial network as an early event. (A) Mitochondrial restructuring and fragmentation occur in the absence of cell shrinkage. Rat1 fibroblasts were infected with Adp20 in the presence of 50 μM zVAD-fmk (to prevent caspase activation and cell detachment) for 20 h, fixed and double stained with anti-tubulin and anti-TOM20 antibodies. (B) Mitochondrial fragmentation occurs prior to activation of BAX and cyt.c release. As in (A) except cells were infected for 25 h and double stained with anti-cyt.c antibody and the active conformation specific anti-BAX-NT antibody (aa 1-21) (Upstate).

4.5.6 p20 induces Drp1 translocation to mitochondria

Recently, fragmentation of mitochondria during staurosporin-induced apoptosis was demonstrated to be dependent on mitochondrial fission mediated by Drp1 (Frank et al., 2001). Therefore, we investigated whether Drp1-dependent fission played a role in the p20 pathway. We began by examining the subcellular localization of Drp1 because GFP-tagged Drp1 was shown to redistribute from a predominately cytosolic location to

predicted sites of division along mitochondrial tubules following treatment with staurosporin (Frank et al., 2001). Figure 4.6A documents that endogenous Drp1 was recruited to mitochondria prior to the onset of mitochondrial fragmentation in Hela cells treated with Adp20. Hela cells were used for this experiment because their mitochondria form long, clearly defined tubules ideal for colocalization studies; the results, however, were also confirmed in Rat1 and H1299 cells. In mock infected Hela cells Drp1 was distributed throughout the cytosol and showed only minor colocalization with mitochondria stained with TOM20 (Fig. 4.6A), likely because Drp1 normally cycles on and off mitochondria continuously (Frank et al., 2001; Smirnova et al., 2001). In contrast, Drp1 showed a strong colocalization with mitochondria in Hela cells infected with Adp20+zVAD-fmk for 17h (Fig. 4.6A, bottom panels). Enlargement of the merged image revealed that Drp1 formed clusters along the surface of mitochondrial tubules prior to the onset of fragmentation. Interestingly, in *C. elegans* similar clusters of GFP-Drp1 on mitochondrial tubules were shown to coincide with future sites of membrane scission (Labrousse et al., 1999). Pretreatment of H1299 cells with TG or expression of b5-BCL-2 reduced the amount of endogenous Drp1 recovered in the mitochondrial fraction following p20 expression (Fig. 4.6B, compare lane 2 to lanes 3 and 4) and inhibited mitochondrial fission (Fig. 4.4C).

4.5.7 Dominant-negative $Drp1_{K38E}$ prevents p20-induced mitochondrial changes

To confirm that p20 mediates its sensitizing effect on mitochondria through Drp1, we examined the effect of a dominant negative Drp1 mutant on p20-induced mitochondrial changes. Mutation of a conserved lysine (K38) in the GTP binding domain

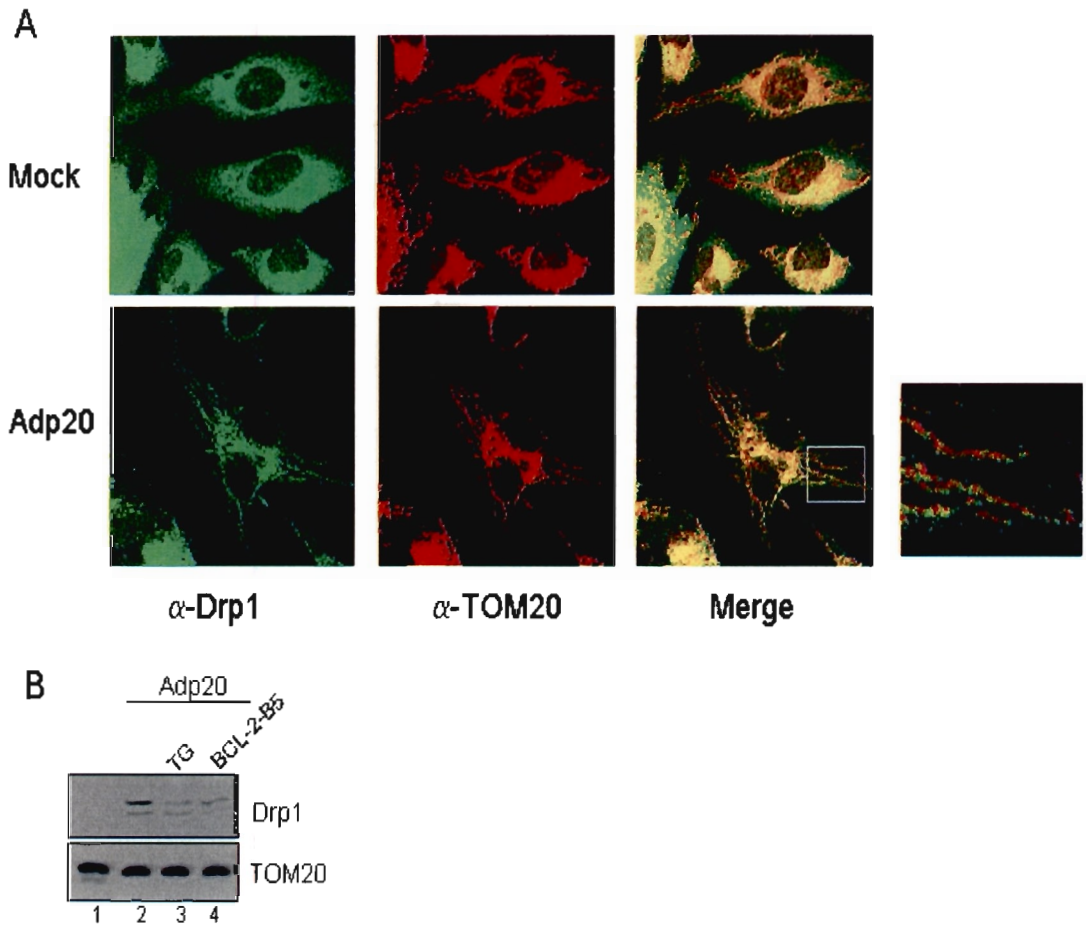


Figure 4.6. Drp1 mediates p20-induced mitochondrial fission. (A) Recruitment of endogenous Drp1 to mitochondria. Hela cells were mock infected (top) or infected with Adp20 (bottom) in the presence of zVAD-fmk and 17 h post-infection cells were fixed, double stained with anti-Drp1 (green) and anti-TOM20 (red) antibodies and imaged by confocal immunofluorescence microscopy. Enlargement of the merged overlay revealed that clusters of Drp1 relocate along mitochondrial filaments prior to the onset of fission. (B) H1299 cells, H1299 cells treated with TG, or H1299 b5-BCL-2 cells were infected with Adp20+zVAD for 18 h and the mitochondrial fraction was isolated and analyzed for the presence of Drp1 by SDS-PAGE and immunoblotting. The blot was reprobed with anti-TOM20 antibody to demonstrate equal protein loading.

of Drp1 is predicted to reduce GTPase activity (Bleazard et al., 1999; Smirnova et al., 1998) and expression of such a mutant inhibits OMM scission (Labrousse et al., 1999). Ectopic expression of CFP-Drp1$_{K38E}$ in Rat1 cells offset the normal balance between mitochondrial fission and fusion and increased the connectivity of mitochondria

compared to untransfected cells or cells transfected with wild type CFP-Drp1 (Fig. 4.7A, top panels, transfected [CFP-positive] cells are indicated by arrows). Overexpression of wild type Drp1 does not induce fission in mammalian cells (Frank et al., 2001; Smirnova et al., 1998) and, accordingly, Rat1 cells transiently transfected with CFP-Drp1 exhibited a normal mitochondrial phenotype and underwent fragmentation in response to Adp20 (Fig. 4.7A). Cells transfected with CFP-Drp1$_{K38E}$, on the other hand, resisted Adp20-induced mitochondrial fission and the highly interconnected network remained intact. As shown in Figure 4.7B and C, CFP-Drp1$_{K38E}$ also inhibited p20-induced cyt.c release and caspase activation. Based on morphological criteria, the recruitment of endogenous Drp-1 to mitochondria, and dominant interference by the Drp1$_{K38E}$ mutant, we conclude that p20 activates Drp1-dependent mitochondrial fission, sensitizing this organelle for cyt.c release.

4.6 Discussion

Engagement of the TNF receptor family of death receptors, including TNF-R1, Fas, Trail-R1, and Trail-R2, with their cognate ligands leads to the recruitment and autoactivation of initiator procaspase-8 (Krammer, 2000). Recent studies implicate that caspase-8 substrates located at distinct cellular loci play key roles in mediating death receptor induced apoptosis. For example, caspase-8 cleavage of the BH3-only molecule BID promotes mitochondrial release of cyt.c and Smac/Diablo (Li et al., 2002b; Yin et al., 1999); cleavage of RIP prevents the activation of NF-κB survival responses (Lin et al., 1999); and cleavage of the cytolinker plectin is important for disassembly of

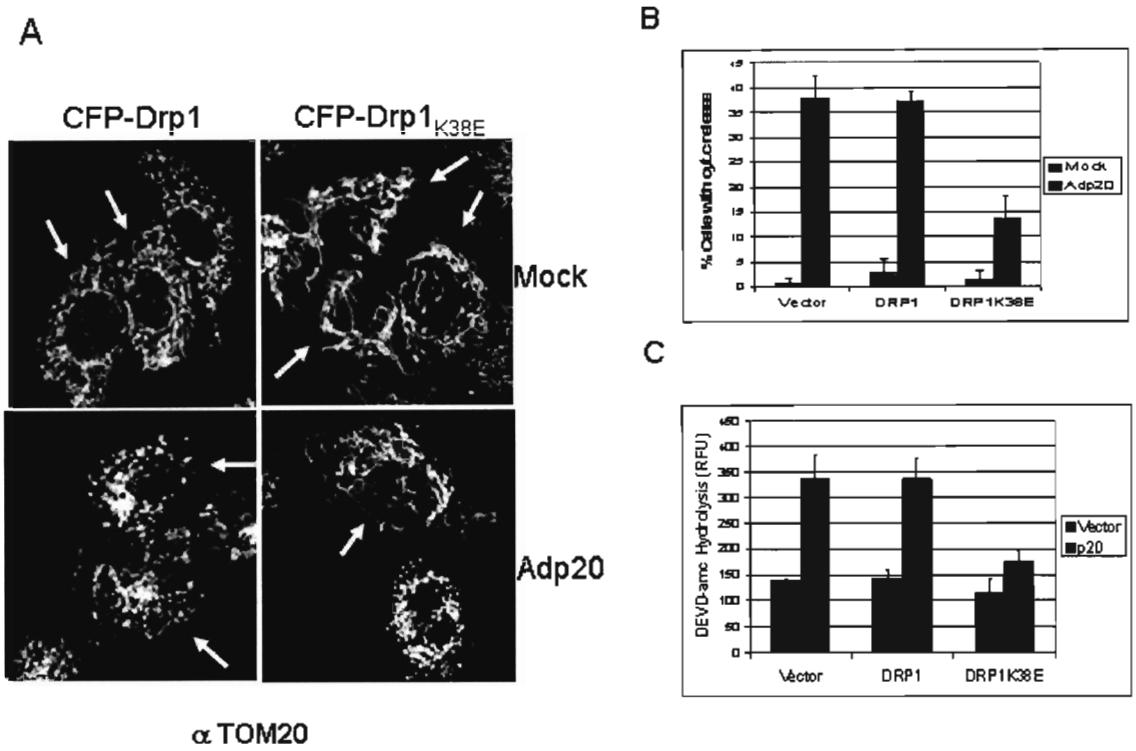


Figure 4.7. Expression of a $Drp1_{K38E}$ dominant negative mutant inhibits p20 induced disruption of the mitochondrial network. (A) Rat 1 Fibroblasts were transiently transfected with CFP-Drp1 or CFP-$Drp1_{K38E}$ then either mock infected or infected with Adp20 in the presence of zVAD-fmk. 24 h post infection cells were fixed, stained with anti-TOM20 and analyzed by fluorescence microscopy. Cells expressing CFP-Drp1 or CFP- $Drp1_{K38E}$ were identified under the cyan filter and are indicated with an arrow. (B) CFP- $Drp1_{K38E}$ inhibits cyt.c release. H1299 cells were treated as in (B) for 36 h and immunofluorescence microscopy was used to assess the distribution of cyt.c in cells positive for CFP fluorescence. Shown is the mean $\pm$ SD of 4 independent experiments. (C) H1299 cells were transiently co-transfected with the indicated constructs and 36 h post transfection cell lysates were collected and processed for DEVDase activity, shown is the mean $\pm$ SD of 3 independent experiments.

microfilaments (Stegh et al., 2000). In this study we investigated the consequence of caspase-8 cleavage of BAP31 at the ER by expressing the pro-apoptotic p20 cleavage fragment in cells using an adenovirus vector. This approach allowed us to isolate and delineate a predicted branch of the death receptor signaling cascade. Specifically, we found that p20 could mediate Ca^{2+}-dependent apoptotic crosstalk between the ER and mitochondria, stimulating mitochondrial fission and sensitization of this organelle to caspase-8-induced cyt.c release.

The importance of BAP31 cleavage during Fas-mediated apoptosis was first highlighted by the observation that expression of the caspase-resistant BAP31 mutant, crBAP31, strongly inhibited apoptotic membrane blebbing and release of cyt.c from mitochondria (Nguyen et al., 2000), suggesting that ER-mitochondrial signaling played a role in this pathway. When we re-examined photographs of mitochondria in crBAP31 cells undergoing Fas-induced apoptosis it was apparent that mitochondrial fragmentation was also strongly inhibited (Nguyen et al., 2000). Thus, full length BAP31 and p20 have opposing functions during Fas-mediated apoptosis, the former inhibiting mitochondrial fission, and egress of cyt.c from mitochondria, and the latter stimulating these events. Importantly, however, p20 operates independently of BAP31 and BAP29 since p20 caused apoptosis in *Bap31*-null and *Bap29,31*-null cells (Fig. 4.2E). Therefore, caspase-8 cleavage of BAP31 converts it from an inhibitor to activator of cell death - a paradigm that has been ascribed to other caspase targets such as BCL-2 (Cheng et al., 1997), BCL-xL (Clem et al., 1998), and RIP (Lin et al., 1999).

Cleavage of BAP31 may contribute to other cell death pathways that signal through caspase-8. For example, we recently reported that BAP31 and BAP29 play a role in the recruitment and activation of procaspase-8L at the ER during E1A-induced apoptosis (Breckenridge et al., 2002). The kinetics of procaspase-8L processing strongly correlated with BAP31 cleavage in response to E1A, suggesting that activated procaspase-8L mat hydrolyze BAP31. The ensuing p20-induced Ca^{2+} release and mitochondrial fission might enhance cyt.c release by other proapoptotic regulators that are activated by E1A, including BIK (Breckenridge and Shore, 2000; Mathai et al., 2002).

Based on studies employing pharmacological modulators of Ca^{2+} signaling and inhibitors of apoptosis and mitochondrial fission, our results suggest that p20 induces an apoptotic

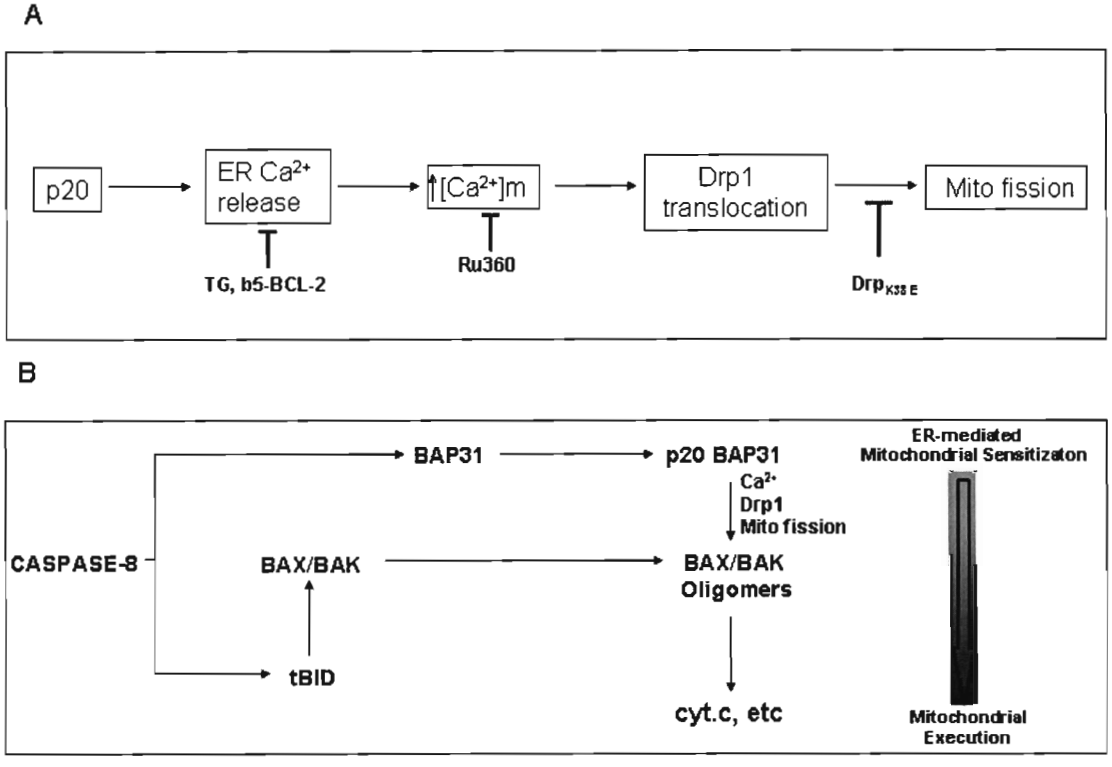


Figure 4.8. A) Proposed mechanism of p20-induced mitochondrial fission. p20 triggers a specific Ca^{2+} signal from the ER that is decoded by mitochondria. Mitochondria, in turn, recruit Drp1, which initiates organelle fission. Lowering ER Ca^{2+} stores by pretreatment with TG or expression of b5-BCL-2, chelating the Ca^{2+} released to the cytosol with BAPTA, blocking mitochondrial uptake of Ca^{2+} with Ru360, or inhibition of Drp1 by expression of $Drp1_{K38E}$ all prevent p20-induced mitochondrial fission. B) A model depicting how in intact cells cleavage of BAP31 at the ER sensitizes mitochondria to caspase-8-driven cyt.c release. Stimulation of Fas leads to caspase-8 dependent processing of BAP31 and BID, generating p20 and tBID. tBID translocates to mitochondria where it induces the oligomerization of BAX/BAK into pores in the OMM. Simultaneously, p20 triggers ER Ca^{2+} release, causing Drp-1 translocation to mitochondria and subsequent organelle fission, enhancing the release of cyt.c to the cytosol.

pathway between the ER and mitochondria (Fig. 4.8A). This is initiated by ER Ca^{2+} release coupled to mitochondrial Ca^{2+} uptake. Importantly, it has been demonstrated that Drp1 recruitment to mitochondria initiates fission (Labrousse et al, 1999; Smirnova et al, 2001). Since either the lowering of ER Ca^{2+} stores, or chelating cytosolic Ca^{2+}, or

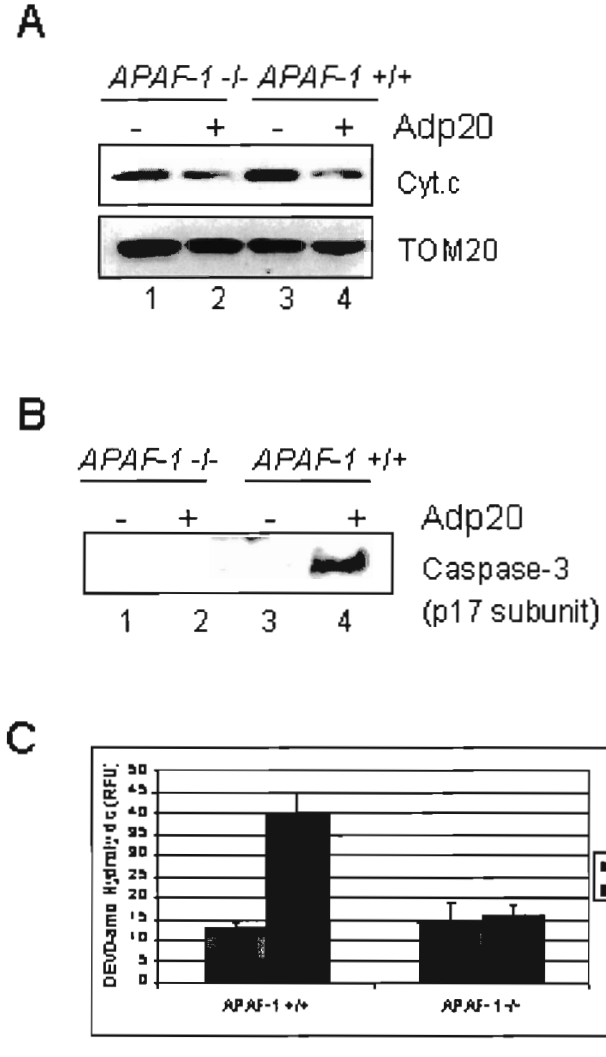
preventing mitochondrial Ca^{2+} uptake all prevented p20-induced fission of mitochondria, it is likely that ER-mitochondrial Ca^{2+} transmission acts upstream of Drp1 translocation in this context. Drp1 recruitment is likely mediated by an OMM receptor protein(s), and this complex likely cooperates with inner mitochondrial membrane reorganizing enzyme(s) to mediate organelle fission (Shaw and Nunnari, 2002). ER and Mitochondrial membranes are often in close proximity and privileged Ca^{2+} exchange between the two organelles has previously been implicated during apoptosis. For example, IP_3 Receptor and Ryanodine receptor mediated Ca^{2+} spikes that modulate mitochondrial metabolism in healthy cells also sensitize mitochondria to proapoptotic stimuli during cell death (Szalai et al., 1999; Hajnoczky et al, 2000) . Moreover, manipulations that increase $[Ca^{2+}]_{ER}$ also increase agonist-induced Ca^{2+} spikes and enhance mitochondrial cyt.c release and apoptosis whereas a lowering of ER Ca^{2+} stores has the opposite effect (Nakamura et al., 2000; Pinton et al., 2001). Modulation of the frequency, amplitude and spatio-temporal pattern of ER Ca^{2+} release during apoptosis may determine how mitochondria respond to Ca^{2+} signals (Berridge et al., 2000; Pacher, et al, 2001). Our results suggest that caspase cleavage of BAP31 may be one mechanism to generate such proapoptotic ER-mitochondrial Ca^{2+}-dependent crosstalk in the Fas pathway.

In isolation, p20 caused ER Ca^{2+} release immediately after its expression and Drp1 redistribution and mitochondrial fission were apparent within several hours of this event but BAX activation, cyt.c release, and caspase activation were significantly delayed. Therefore, in the absence of a parallel BH3-dependent hit, mitochondria undergo fission in response to p20 and probably remain in a fragmented state (without releasing cyt.c) until a second signal responds and activates BAX/BAK. In a normal death receptor signaling context, however, simultaneous processing of BAP31 and BID

by caspase-8 would be predicted to mount a dual attack on mitochondria, with p20 causing mitochondrial fission and tBID inducing cristae remodeling and activation of BAX and BAK (Scorrano et al., 2002) (Fig. 4.8b). Apoptotic cristae remodeling and mitochondrial fission may be intimately linked since cristae reorganization occurs during normal fission and fusion events in healthy cells (Bereiter-Hahn and Voth, 1994; Shaw and Nunnari, 2002) and mitochondrial fission is a requisite for cyt.c release (Frank et al., 2001). A "two hit" model in which an ER–mitochondrial Ca^{2+} signal and a direct mitochondrial insult synergize to promote the mitochondrial phase of apoptosis likely functions in other apoptosis pathways (Pinton et al., 2001; Szalai et al., 1999). Of note, tBID was reported to induce caspase-independent mitochondrial fragmentation on its own (Li et al., 1998) and, therefore, p20 signaling may not be an obligate requirement for cyt.c release on the death receptor pathway but rather a sensitizer of this event. Indeed, the combined actions of p20 and tBID could cooperate in vivo since p20 strongly enhanced the ability of caspase-8 to promote cyt.c release without affecting the extent of BID cleavage (Fig. 4.3). This duality in signaling may be particularly relevant in physiological situations where apoptotic stimuli are sub-optimal or are countered by opposing survival signals and the fate of the cell hinges upon the balance of proapoptotic and antiapoptotic signals received by mitochondria.

4.7 Supplemental Data

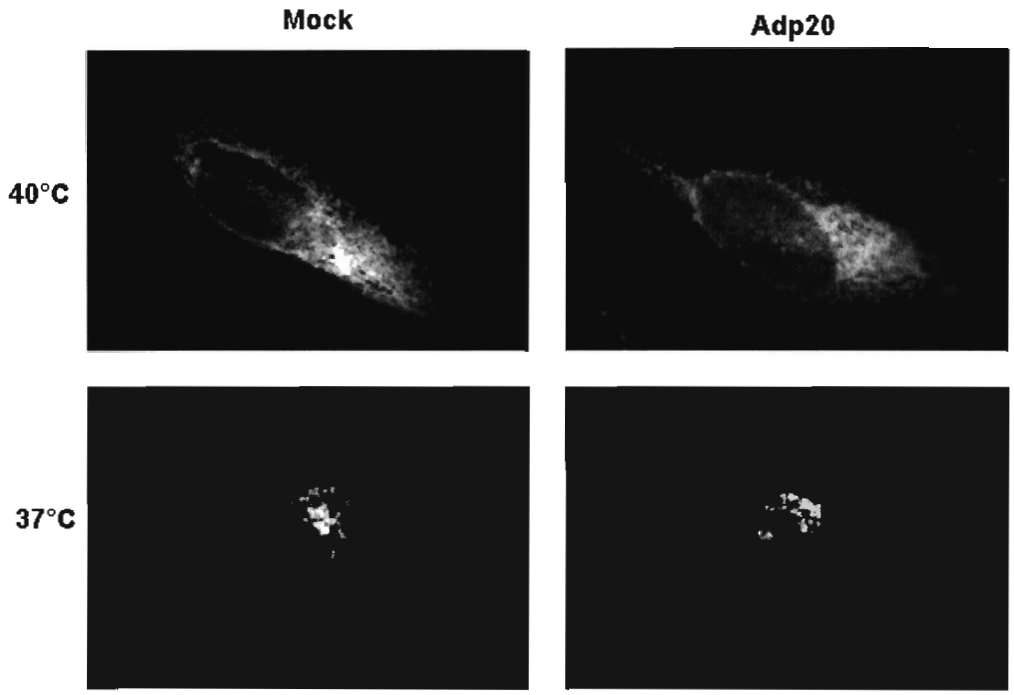
Supplemental Figure 1



Supplemental Figure 4.1. p20 induces cyt.c release but not caspase activation in *APAF-1 -/-* mouse embryonic fibroblasts (MEFs). (A) *APAF-1 -/-* or *APAF-1 +/+* MEFs (Yoshida et al., 1998) were mock infected or infected with Adp20 for 40 h and the presence of cyt.c in the mitochondrial fraction was

assessed by immunoblotting. (B) As in (A) except cell lysates were tested for caspase-3 processing using an antibody specific for the active p17 subunit. (C) As in (B) except cell lysates were tested for DEVDase activity (mean and standard deviation of 3 independent experiments)

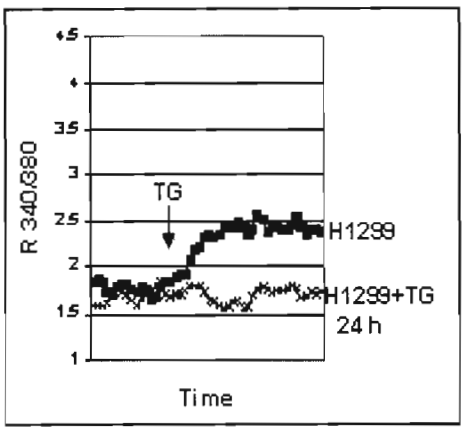
Supplemental Figure 2



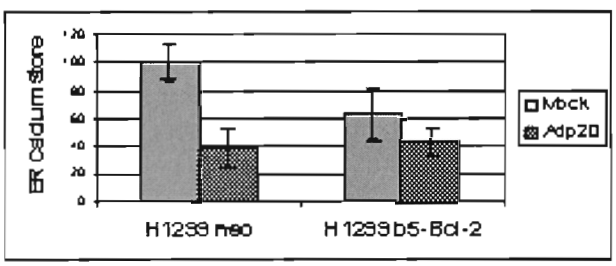
Supplemental Figure 4.2. Adp20 infection does not affect ER-Golgi trafficking. Hela cells were transfected with temperature-sensitive VSV-G-EGFP for 20 h, then mock infected or infected with Adp20 for an additional 22h. Cells were cultured at 40°C to prevent the transport of VSV-G-EGFP out of the ER, and where indicated cells were transferred to 37°C for 1 h to promote VSV-G-EGFP trafficking through the secretory pathway.

Supplemental Figure 3

A



B



Supplemental Figure 4.3. A) Lowering of resting ER Ca^{2+} stores with TG. Left, H1299 cells were left untreated or incubated in presence of 50 nM TG and zVAD-fmk for 24 h and the ER Ca^{2+} content was assessed as decribed in Materials and Methods. Shown is a representative trace. B) overexpression of b5-Bcl-2 lowers the ER Ca^{2+} content. ER Ca^{2+} stores were assessed in mock or Adp20 + zVAD-fmk (24 h) treated H1299-neo cells or in H1299 b5-Bcl-2 cells (mean $\pm$ SD of 4 experiments). Immunofluorescence microscopy confirmed that b5-Bcl-2 was located exclusively at the ER (data not shown).

4.8 Acknowledgements

We are very grateful to H. McBride for the Drp1 expression vectors and helpful discussions, P. Clemons for FK1012Z, M. Germain for H1299 b5-BCL-2 cells, T. Mak for *APAF-1*-null MEFs, and J. Lynch and M. Michalack for Fura-2 protocols and anti-calreticulin antibody. D.G.B. is a recipient of a Canadian Institute of Health Research Doctoral award. This work was supported by the National Cancer Institute of Canada, and the Canadian Institutes of Health Research.

Chapter 5

Intact BAP31 Inhibits Fas-Induced Release of Calcium from the Endoplasmic Reticulum and Cytochrome c from Mitochondria in Intact Cells

5.1 Rationale

The experiments in chapter 4 revealed that the caspase-cleavage product of BAP31 can drive a Ca^{2+} signal between the ER and mitochondria, which ultimately sensitizes mitochondria to caspase-8-induced cyt.c release. It is plausible, therefore, that the protective effect of crBAP31 against cell death is primarily due to a block in this ER-mitochondrial Ca^{2+} signal. In this chapter, we investigated whether crBAP31 does indeed influence Fas-induced mobilization of ER Ca^{2+} stores and the effect of crBAP31 on the specific sequence of biochemical events that leads to cyt.c release from mitochondria on the Fas pathway. Another important question that was addressed in chapter 5 is whether crBAP31 exerts its protective effect by inhibiting the proapoptotic function of p20 (derived from cleavage of endogenous BAP31), or whether full-length (intact) BAP31 has a bonafide antiapoptotic function prior to its cleavage and conversion to the proapoptotic p20 fragment.

5.2 Abstract

BAP31 is a polytopic integral protein of the endoplasmic reticulum (ER) membrane and, like BID, is a preferred substrate of caspase-8. Upon Fas/CD95 stimulation, BAP31 is cleaved within its cytosolic domain, generating a pro-apoptotic p20 fragment that triggers Ca^{2+} transfer between the ER and mitochondria and stimulates large-scale fission of the mitochondrial network. In human KB epithelial cells expressing the caspase-resistant mutant crBAP31, Fas-induced release of ER Ca^{2+} stores was inhibited, but a late and sustained elevation in cytosolic Ca^{2+} was unaffected. In crBAP31 cells, Fas stimulation still resulted in cleavage of BID and insertion of BAX into

mitochondrial membrane, but subsequent oligomerization of BAX and BAK, egress of cyt. c to the cytosol, and apoptosis were impaired. *Bap31*-null mouse cells expressing crBAP31 cannot generate the endogenous p20 BAP31 cleavage product, yet crBAP31 conferred resistance to cellular condensation and cyt. c release in response to activation of ectopic FKBP-casp8 by FK1012Z. Full length BAP31, therefore, is an inhibitor of these caspase-8 initiated events. Taken together out results suggest that caspase cleavage of BAP31 converts it from an inhibitor to activator of Ca^{2+}-dependent proapoptotic cross-talk between the ER and mitochondria.

5.3 Introduction

An emerging model for the regulation of apoptosis posits that Bcl-2 family members regulate and integrate upstream death signals that ultimately cause a breach in the mitochondrial outer membrane, releasing factors that contribute to the cell's demise (Bouillet and Strasser, 2002; Korsmeyer et al., 2000). Although multiple extrinsic and intrinsic pathways likely converge on mitochondria to achieve this end, the coupling of mitochondrial responses to upstream stimuli has been most intensively investigated for apoptosis initiated by cell surface death receptors. Activation of the Fas/CD95 signaling complex, for example, causes recruitment and processing of the two major isoforms of procaspase-8, -8/a and -8/b (Scaffidi et al., 1997). The resulting caspase-8 holoenzyme can then initiate a downstream cascade of events, including direct processing of effector procaspases such as procaspase-3, at least in certain contexts (Huang et al., 2000; Scaffidi et al., 1998; Scaffidi et al., 1999). An additional target of caspase-8, however, is the pro-apoptotic BH3-only member of the Bcl-2 family, BID (Gross et al., 1999b; Li et al., 1998;

Luo et al., 1998). Caspase-8 cleavage of BID generates tBID, which inserts into the mitochondrial outer membrane where its exposed BH3 death ligand drives the release of cyt.c from the organelle (Wei et al., 2000). In addition to recruiting BAX into the outer membrane bilayer (Eskes et al., 2000; Ruffolo et al., 2000), mitochondrial tBID induces intramembrane oligomerization of BAX (Antonsson and Martinou, 2000; Eskes et al., 2000) and BAK (Wei et al., 2000; Wei et al., 2001), causing these pro-apoptotic Bcl-2 members to form a proposed conduit for cyt. c egress from the organelle (Korsmeyer et al., 2000). Once released, cyt.c becomes an integral constituent of the apoptosome, resulting in further amplification of a caspase cascade (Budihardjo et al., 1999). Consistent with an essential but redundant role for BAX and BAK in these tBID-dependent events, *Bax* and *Bak* double knock out mouse cells, but not single knock outs, remain refactory to ectopic tBID . In view of the fact that several other caspase-8 preferred substrates have been identified (Lin et al., 1999; Nguyen et al., 2000; Stegh et al., 2000), however, an obvious question concerns the contribution they might make to Fas signaling pathways.

Human BAP31 is a 28 kDa polytopic integral protein of the endoplasmic reticulum (ER) and part of a large BAP hetero-oligomeric complex that includes the related BAP29 protein and connections to actomyosin (Breckenridge et al., 2002; Kim et al., 1994; Ng et al., 1997; Nguyen et al., 2000). In addition to its role in the recruitment and regulation of the novel procaspase-8 isoform, procaspase-8L, in response to apoptotic signaling by oncogenic E1A (Breckenridge et al., 2002), BAP31 itself is a preferred substrate for caspase-8 (Ng et al., 1997). Cleavage occurs at two identical sites (AAVD.G) in the human protein that flank the DECC (death effector-like coiled coil) domain within the cytosolic disposed COOH-terminal tail of BAP31 (Ng et al., 1997;

Nguyen et al., 2000). As a first step to investigate the contribution of BAP31 cleavage during apoptosis, we recently established a KB epithelial cell line that expresses caspase-resistant (cr) BAP31, in which the caspase-recognition asp residues have been mutated to ala. Caspase-8 activation that is independent of BAP31 in these cells is then achieved by stimulating the Fas death-inducing signaling complex (DISC) (Nguyen et al., 2000). In this system, crBAP31 provided a remarkably pleiotropic resistance to Fas-mediated cytoplasmic apoptosis despite the fact that it had little influence on processing of procaspases-8/a and -8/b and activation of downstream effector caspases. In contrast, apoptotic membrane blebbing/fragmentation and redistribution of actin were strongly inhibited, the cells retained a near normal morphology, and irreversible loss of cell growth potential following removal of the Fas stimulus was delayed (Nguyen et al., 2000). Preservation of full length BAP31 in the face of Fas stimulation also restrained the release of cyt.c from mitochondria (Nguyen et al., 2000). These results suggest, therefore, that cleavage of BAP31 during Fas-mediated cell death might regulate a proximal step that impacts mitochondrial function and cytoplasmic apoptosis.

Mobilization of ER Ca^{2+} stores is an early hallmark response to many death signals, including death receptor stimulation, where it contributes to multiple aspects of apoptosis progression (Jayaraman and Marks, 1997; McConkey and Nutt, 2001; Pu and Chang, 2001; Scoltock et al., 2000). Large and sustained global elevations in cytosolic Ca^{2+} have also been observed during apoptosis (McConkey and Nutt, 2001). Altered cellular Ca^{2+} homeostasis may regulate a number of apoptotic processes, including the activation of signal transducing enzymes such as calpains and calcineurin (Ferrari et al., 2002; Wang, 2000). In addition, privileged Ca^{2+} transport between the ER and mitochondria contributes to sensitization of mitochondria to BH3-dependent cyt.c release

(Csordas et al., 2002; Hajnoczky et al., 2000a). Since BAP31 is a caspase-8 target located at the ER, its cleavage may thus impact ER Ca^{2+} homeostasis, as well as other possible pathways of the ER that regulate apoptosis. Consistent with this hypothesis, we recently reported that the p20 caspase cleavage product of BAP31 can trigger ER Ca^{2+} release, which is coupled to increased mitochondrial Ca^{2+} uptake and subsequent large scale fission of mitochondria (Breckenridge et al., 2003). Here, we report that crBAP31 is infact an inhibitor of Fas-induced mobilization of ER Ca^{2+} stores. Furthermore, using *Bap31*-deleted mouse cells we provide evidence that full length BAP31 operates as a direct inhibitor of caspase-8-initiated events rather than operating to sequester the p20 pro-apoptotic cleavage product of BAP31, with which it otherwise interacts.

5.4 Materials and Methods

General – The routine procedures used in this study for measuring apoptotic cell death by microscopic examination of uptake of trypan blue, generation of cellular fractions, SDS PAGE, immunoblotting and development of blots by enhanced chemoluminescence, and assays to measure release of cytochrome c from mitochondria, have been documented in earlier publications (Goping et al., 1998; Ng et al., 1997; Nguyen et al., 2000; Ruffolo et al., 2000).

Antibodies – The following antibodies were used in this study: chicken anti-human BAP31 (Ng et al., 1997) and anti-human TOM20 (Goping et al., 1995); rabbit polyclonal antibody raised against recombinant A4 C-terminus (aa 132-152); rabbbit anti-human

BAK (Upstate); mouse anti-Flag (Sigma); rabbit polyclonal antibodies against the p18 catalytic subunit of caspase-8 (gift from D. Nicholson) and γ-actin (gift from P. Braun); rabbit polyclonal antibody raised against the p15 caspase cleavage product of recombinant BID and purified by affinity selection; mouse monoclonal antibody against pigeon cytochrome c (Pharmingen); rabbit antibody against human BAX aa 1-21 (Upstate) or aa 1-20 (Santa Cruz).

Cells and Transfections - Human KB epithelial cell lines expressing wt BAP31-Flag or crBAP31-Flag were created and cultured as described previously (Nguyen et al., 2000). Differentiated Bap31-null mouse ES cells were created as described (Breckenridge et al., 2002). Stable expression of crBAP31-Flag in differentiated Bap31-null cells was established by transfection (Nguyen et al., 2000) using lipofectAMINE 2000 (Gibco BRL , following manufactures protocol) and cells cultured in KNOCKOUT D-MEM™ (Gibco BRL) in the presence of 150 μg/ml G418. Clones were isolated that expressed crBAP31-Flag at levels similar to the level of Bap31 expressed in wt cells.

Activation of FKBPcasp8 by the Dimerizing Compound FK1012z - The expression vectors MF_{pk}3FLICE (Muzio et al., 1998) and pcDNA3-GFP were transiently co-transfected into differentiated *Bap31*-null or wt mouse ES cells. After 24 h, solvent (mixture of 50% ethanol and 50% DMSO) containing or lacking FK1012z (final concentration, 1.0 nM), a derivative of FK1012 prepared by olefin metathesis using a modification of the procedure described by Diver and Schreiber (Liberles et al., 1997),

was added. At the indicated times, cells were collected and 25 μg of total lysates were subjected to SDS PAGE and immunoblotting using anti-HA antibody.

Immunofluorescence - For stable cell lines, cells were grown on coverslips for 24 hours. They were treated with 0.5 μg/ml α-human Fas activating antibody (Upstate) in the presence of 10 μg/ml cycloheximide for the indicated times. Cells were fixed in 4% paraformaldehyde, and immunostained as described elsewhere (Nguyen et al., 2000). Cells were visualized by confocal microscopy or by Nikon TE-FM Epi-fl microscopy. For analysis of the *Bap31*-null cells or their derivatives, the cells were grown on glass coverslips for 24 h. They were transfected , using lipofectAMINE 2000 , with 0.2 μg pCMS-EGFP (Clonetech) and 0.8 μg MF_{pk}3FLICE (Muzio et al., 1998) per coverslip. 24 h later , they were treated with or without FK1012z and the cells subsequently fixed at the indicated times with 4% paraformaldehyde . Similarly , Cos 1 cells were transfected with 0.5μg of A4-HA and 0.5μg of BAP31-Flag per coverslip for 24 hours before fixing and immunostaining . Nuclei were stained with DAPI and fluorescence analyzed by confocal microscopy or by Nikon TE-FM Epi-fl microscopy.

Ca^{2+} measurements - ER Ca^{2+} stores were evaluated exactly as in chapter 4. Indo-1 AM was used to measure Fas-induced increase in cytosolic Ca^{2+} by flow cytometry. Breifly, cells were collected at the indicated times following stimulation with Fas/CHX, washed once in PBS, then loaded with 2 μM Indo-1 in EBSS for 30 min at 37 °C. Cells were then washed three times in EBSS and calcium levels were measured as the ratio of Indo-1 fluorescence emission at 400 nm and 480 nm at an excitation wavelength of 365 nm.

5.5 Results

5.5.1 crBAP31

The schematic in Fig. 5.1A depicts the topology of human BAP31 in the membrane of the ER and denotes the two identical caspase recognition sites (AAVD.G) flanking the cytosolic disposed DECC domain in the protein (Ng et al., 1997; Nguyen et al., 2000). Cleavage at these sites generates the products p27 and p20 (Fig. 5.1B). In contrast to the human protein, murine BAP31 lacks the distal caspase cleavage site and generates only the p20 product during apoptosis (data not shown). Human crBAP31-Flag was created by mutating the two caspase recognition aspartate residues to alanine, and inserting the Flag epitope immediately upstream of the canonical KKEE ER retrieval signal located at the extreme COOH-terminus of the protein. Human KB epithelial cell lines were established that stably express crBAP31-Flag at levels 2- to 3-fold higher than that of the endogenous BAP31 protein (Nguyen et al., 2000). Upon stimulation of these crBAP31-Flag cells with 0.5 μg/ml agonistic antibody against Fas/CD95, in the presence of 10 μg/ml cyclohexamide (CHX) to enhance sensitivity to Fas activation (Scaffidi et al., 1999), caspases, including caspase-8 and caspase-3, are activated. As a result, caspase targets such as endogenous BAP31 and poly(ADP ribosyl-) polymerase (PARP) are cleaved (Nguyen et al., 2000).

As documented in Fig. 5.1B, the time course for this Fas-induced cleavage of endogenous BAP31 was very similar for wt parental KB cells and KB cells expressing crBAP31-Flag, indicating that activity of upstream caspase is similar in the two cell types. Moreover, the two cell types express equivalent levels of Fas receptor (as determined by immunoblot, not shown) and, following stimulation, process

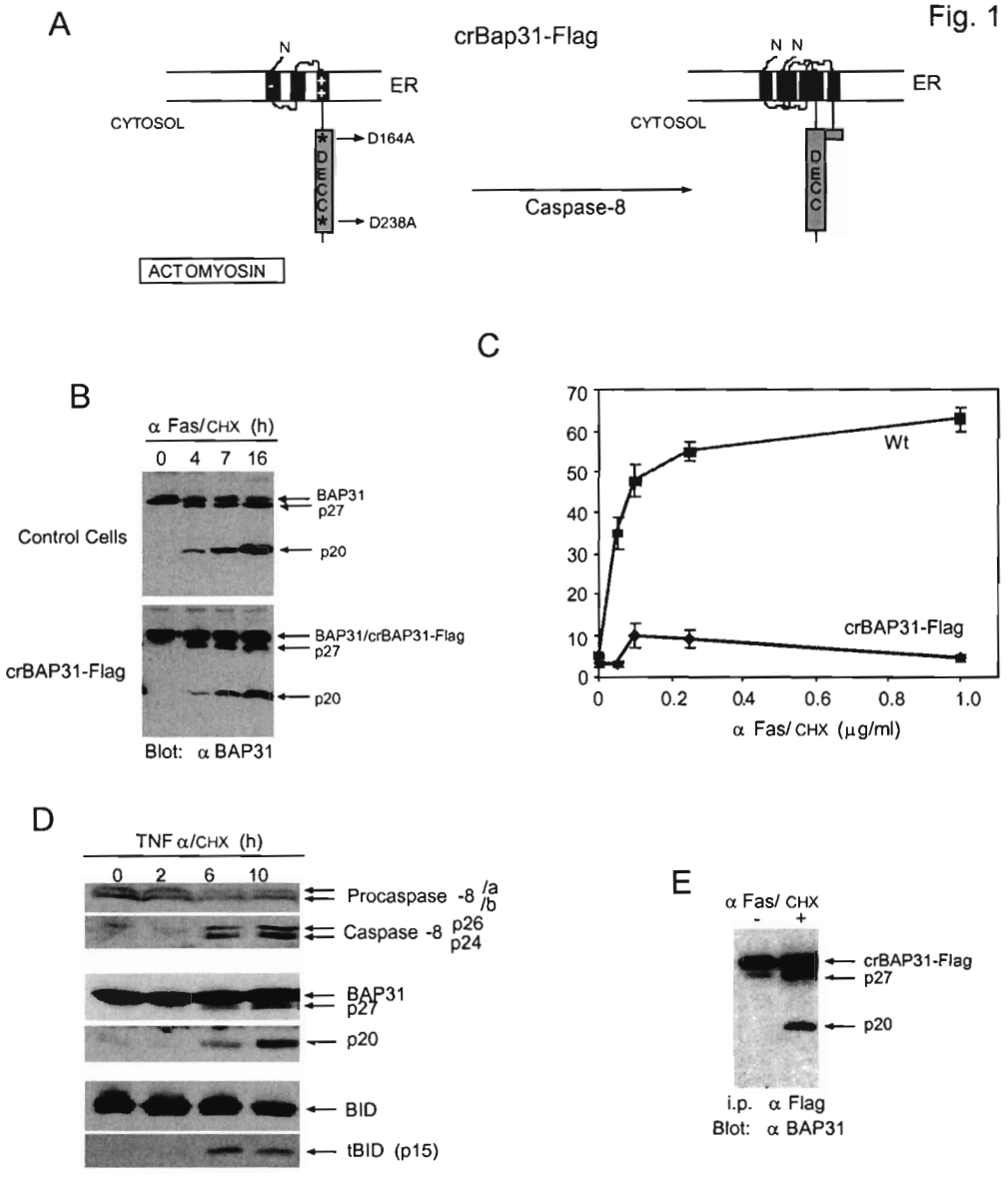


Figure 5.1. Cleavage of BAP31 following stimulation of death receptors. (A) Topology of BAP31 in the ER membrane. The presence of charged residues in the predicted transmembrane segments are indicated by + and -. To create crBAP31, the caspase-recognition asp (D) residues were converted to ala (A) (asterisks); the Flag epitope sequence was inserted immediately upstream of the KKEE ER retrieval signal located at the COOH-terminus. DECC, weak death effector and overlapping coiled coil domain. Hetero-oligomer of ectopic p28 crBAP31-Flag and endogenous p20 BAP31 is shown on the right. (B) crBAP31 resists Fas-induced caspase cleavage. Wt parental (control) KB cells (upper panel) or KB cells expressing crBAP31-Flag (lower panel) were stimulated with 0.5 μg/ml anti-Fas (α Fas) antibody in the presence of 10 μg/ml cyclohexamide (CHX) for the indicated times, and whole cell lysates were subjected to SDS PAGE, immunoblotted with chicken anti-BAP31 (α BAP31) antibody. (C) Parental KB cells (Wt) or KB cells expressing crBAP31-Flag were stimulated for 7 h with the indicated concentrations of Fas in the presence of 10 μg/ml CHX and analyzed for apoptotic index (% trypan blue positive apoptotic cells ± standard deviation for 3 independent determinations). (D) MCF-7 cells were treated with TNFα/CHX and at the indicated times aliquots of whole cell lysates containing equivalent amounts of protein were subjected to SDS PAGE and immunoblotted with antibodies (α) against p18 caspase-8 (upper two panels, showing

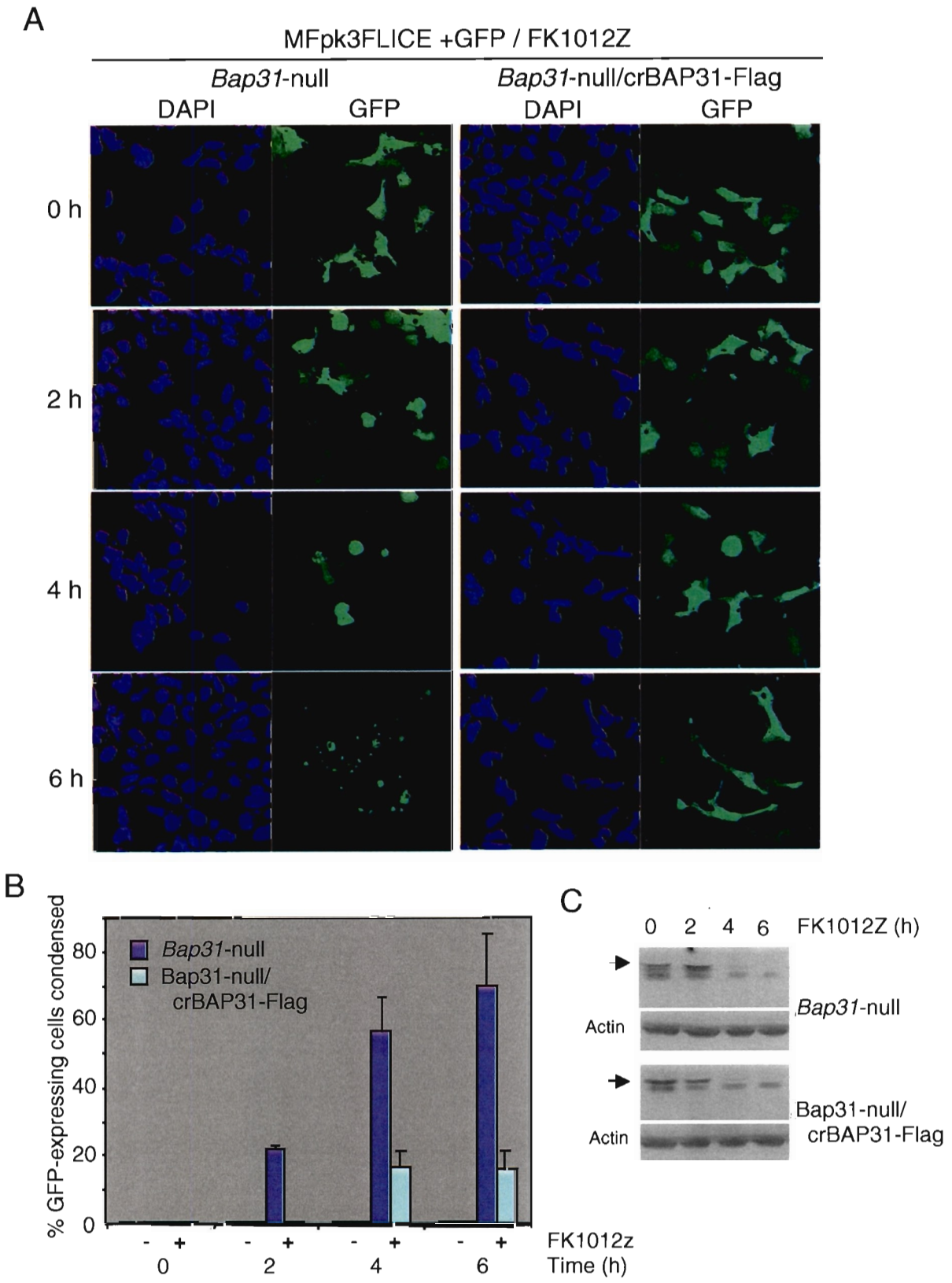
unprocessed procaspase-8/a and -8/b and the processing intermediates p24 and p26), anti-BAP31, which detects full length BAP31 as well as the p27 and p20 cleavage products (middle two panels), and anti-BID, which also weakly detects p15 tBID (lower two panels). (E) KB cells expressing crBAP31-Flag were stimulated with or without 0.5 μg/ml anti-Fas (α Fas) antibody in the presence of 10 μg/ml CHX for 7 h and total cell lysates subjected to immunoprecipitation with anti-Flag antibody. The resulting precipitates were resolved by SDS PAGE and immunoblotted with chicken anti-BAP31.

procaspase-8/a and –8/b to a similar extent (Nguyen et al., 2000). In contrast to endogenous BAP31, however, crBAP31-Flag was not cleaved (Fig. 5.1B) and cells expressing crBAP31-Flag resisted apoptotic membrane blebbing (Nguyen et al., 2000) and loss of cell viability as assessed by the uptake of trypan blue (Fig. 5.1C), despite the fact that caspases are active in these cells. Caspase cleavage of the endogenous BAP31 in crBAP31-Flag-expressing cells generated the pro-apoptotic fragment, p20 BAP31 (Fig. 5.1B); however, this truncated p20 BAP31 product associated with excess crBAP31-Flag (Fig. 5.1E) and remained non-toxic (unpublished).

In vitro, BAP31 exhibits a marked preference for cleavage by caspase-8 rather than by caspase-3 (Ng et al., 1997). To extend this to a cellular context, MCF-7 mammary epithelial cells lacking caspase-3 were stimulated with TNFα, which leads to high levels of caspase-8 activity but low or insignificant activation of caspases-1, -2, -3, -5, -6, -7, -9, or -10 (Janicke et al., 1998; Stegh et al., 2000). As shown in Fig. 5.1D, TNFα stimulation of MCF-7 cells caused procaspase-8/a and -8/b to be processed, which correlated with the detectable cleavage of both BAP31 and BID. Thus, caspase-8 generates BAP31 and BID cleavage products as early events following TNFα receptor activation in intact MCF-7 cells.

5.5.2 Full Length BAP31 Is an Inhibitor of Caspase-8-Induced Release of cyt.c from Mitochondria in Intact Cells

To test the ability of full length BAP31 to directly inhibit the apoptotic pathway initiated by caspase-8, crBAP31-Flag was reconstituted in mouse cells deleted of the *Bap31* gene (Breckenridge et al., 2002; S. Kuppig and M. Reth, unpublished), and the cyt. c release pathway initiated by chemical activation of caspase-8. Since these *Bap31*-null cells do not express endogenous (i.e., cleavable) BAP31, their resistance to caspase-8 cannot be explained by the ability of crBAP31 to sequester the pro-apoptotic p20 cleavage product of endogenous Bap31 (Fig. 5.1A,E). The response of *Bap31*-null/crBAP31-Flag cells was compared to that of *Bap31*-null cells as control; again, the latter cannot generate the pro-apoptotic cleavage product of endogenous Bap31, which could contribute to the outcome. MF_{pk}3FLICE, a vector expressing a fusion protein in which triplicate copies of FKBP and an HA tag replace the prodomain of procaspase-8 (Muzio et al., 1998), was transiently transfected into *Bap31*-null and *Bap31*-null/crBAP31-Flag cells, together with vector expressing Green Fluorescent Protein (GFP). 24 h later, cells were treated with the FKBP dimerizing chemical, FK1012Z, which initiates aggregation and autoactivation of the casp8 fusion protein (Muzio et al., 1998), or the cells were treated with vehicle alone. Commensurate with processing of FKBP-caspase-8 (Fig. 5.2C), co-transfected GFP-expressing *Bap31*-null cells lacking crBAP31-Flag rapidly condensed whereas those expressing crBAP31-Flag resisted this apoptotic response (Fig. 5.2A and 5.2B). Moreover, cyt.c remained punctate in cells expressing crBAP31 and treated with FK1012z, whereas it was found diffuse throughout the apoptotic cells lacking crBAP31 (Fig. 5.2D and 5.2E). In both cell types, however, similar levels and processing of ectopic MF_{pk}3FLICE in response to FK1012Z were


A
MFpk3FLICE +GFP / FK1012Z
Bap31-null
Bap31-null/crBAP31-Flag
DAPI
GFP
DAPI
GFP
0 h
2 h
4 h
6 h
B
% GFP-expressing cells condensed
0
20
40
60
80
Bap31-null
Bap31-null/
crBAP31-Flag
- + - + - + - +
0 2 4 6
FK1012z
Time (h)
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0 2 4 6 FK1012Z (h)
Bap31-null
Actin
Bap31-null/
crBAP31-Flag
Actin

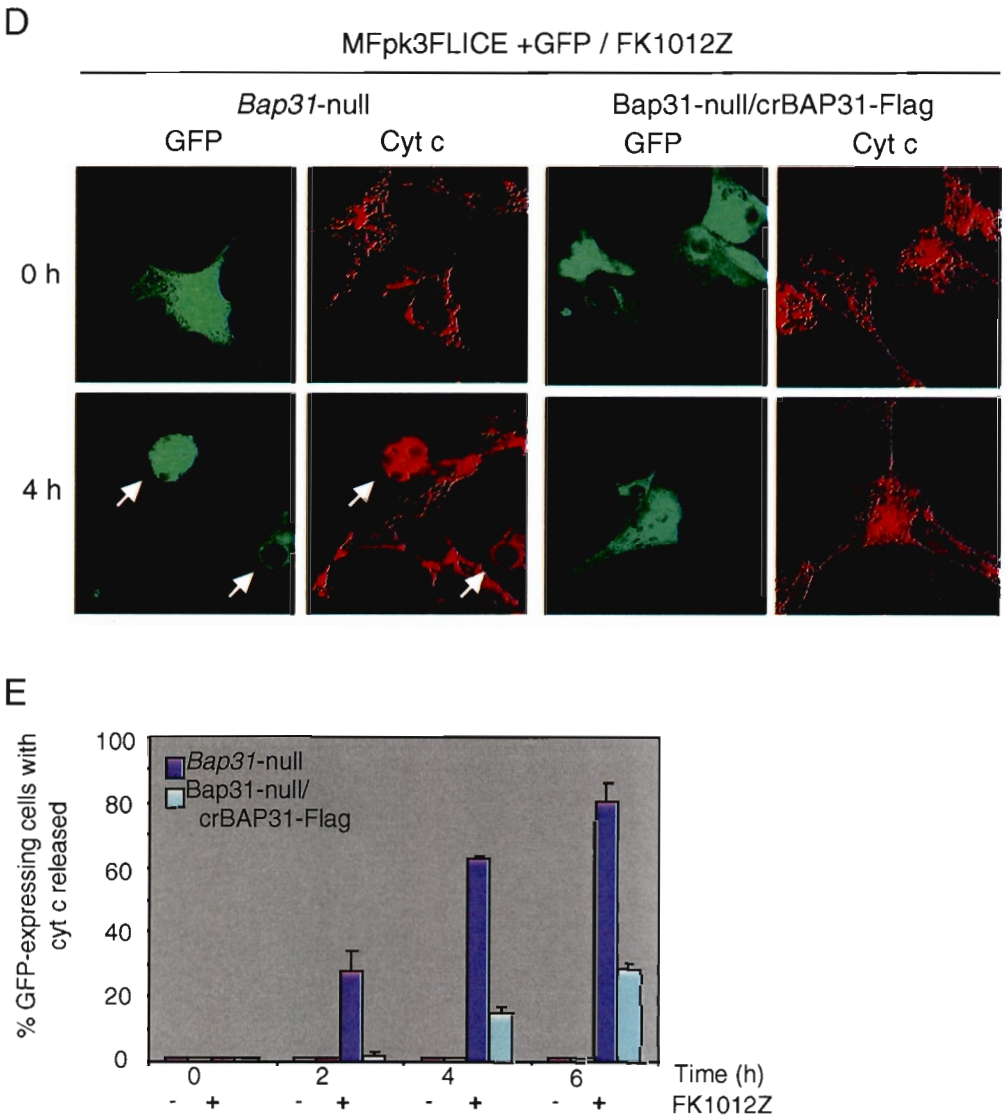


Figure 5.2. crBAP31 inhibits caspase-8-induced cellular condensation and cytochrome c release from mitochondria. (A) *Bap31*-null mouse cells or *Bap31*-null/crBAP31 cells were transfected with MF_{pk}3FLICE and pCMS-EGFP for 24 hours after which time FK1012z (or vehicle alone, not shown) was added. At the indicated time points, cells were fixed and stained with DAPI. Visualization of the fluorescence for DAPI (blue) or GFP (green) was carried out using confocal microscopy. (B) After treatment with FK1012z or vehicle alone, green fluorescent cells that had a condensed morphology were scored and expressed as % of total GFP positive cells (± standard deviations from 3 independent determinations). (C) Immunoblotting of total cellular lysate with anti-HA antibody to detect the HA tagged MFpk3FLICE at the indicated time points following addition of FK1012z. Equivalent gel loading was determined by probing the blot with anti-γ-actin antibody. (D) Similar to (A) except that cells were stained with Alexa-594 conjugated anti-cyt.c, and green (GFP) and red (cytochrome c) fluorescence visualized by Nikon TE-FM Epi-fl microscopy. (E) GFP-expressing cells that showed diffuse cyt.c staining (released, see arrows in (D)) after treatment of cells with FK1012z or vehicle alone were scored and expressed as % of total green cells (± standard deviations from 3 independent determinations).

observed (Fig. 5.2C). Collectively, the results show that full-length (uncleaved) BAP31 inhibits caspase-8 driven cellular condensation and release of cyt.c from mitochondria. As expected, Bap31-null cells expressing wt BAP31, which was cleaved and generated p20 following activation of caspase-8, were fully sensitive to the caspase-8 initiated events (not shown).

5.5.3 crBAP31 Inhibits Fas-induced Mobilization of ER Ca^{2+} Stores.

Since caspase cleaved BAP31 can promote ER Ca^{2+} release, we next tested whether crBAP31 can inhibit this process in response to Fas stimulation. To that end, parental KB cells or crBAP31 cells were stimulated with anti-Fas antibody and the ER Ca^{2+} content was measured. ER calcium stores were defined as the pool of Ca^{2+} that is rapidly released to cytosol in response to treatment of intact cells with Thapsigargin (TG), an inhibitor of ER SERCA pumps. The Ca^{2+}-sensitive fluorescent dye Fura-2 was used to measure the resulting increase in cytosolic Ca^{2+}. crBAP31 did not alter the resting level of ER Ca^{2+} content in healthy cells. As shown in Figure 5.3A, however, crBAP31 restrained the release of ER Ca^{2+} to the cytosol, which in wt cells occurred early following Fas stimulation. crBAP31 did not, however, inhibit global raises in cytosolic Ca^{2+}, which occurred at a later time following Fas-stimulation (Fig. 5.3B). Therefore, caspase cleavage of BAP31 selectively promotes ER Ca^{2+} release during Fas mediated apoptosis.

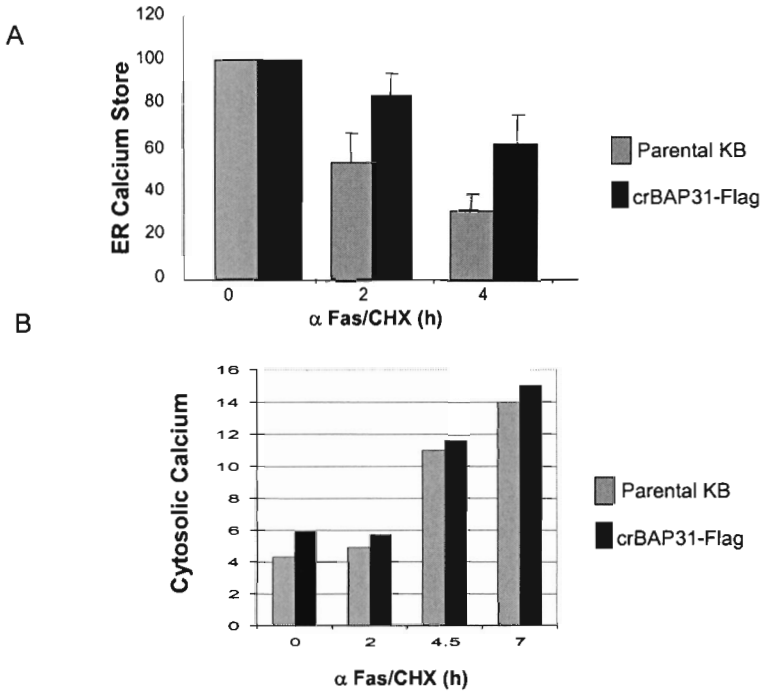


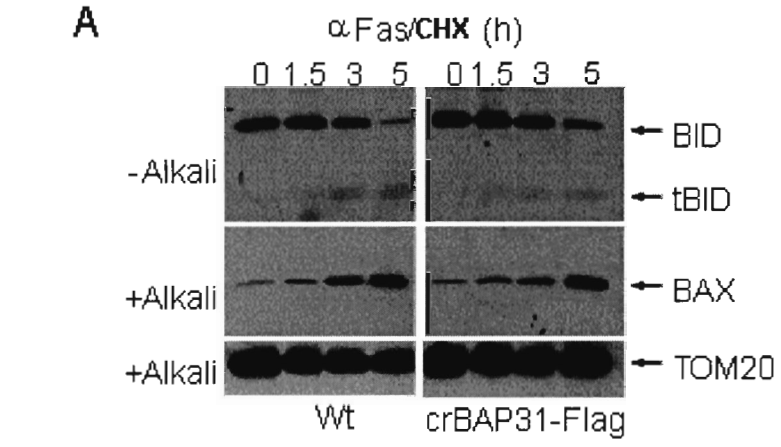
Figure 5.3. crBAP31 impairs Fas-mediated ER Ca^{2+} release but not later elevations in cytosolic Ca^{2+}. A) parental KB (wt) cells or KB cells stably expressing crBAP31-Flag were stimulated with anti-Fas for the indicated times, and the ER Ca^{2+} stores determined by the sudden difference in Fura-2 fluorescence recorded after adding TG to the cells (see Materials and Methods). The results represent relative values and the average and S.D. obtained from 5 independent experiments. B) As in A) except cells were loaded with Indo-1and relative fluorescence levels were measured by flow cytometry. Shown is a representative from three independent experiments.

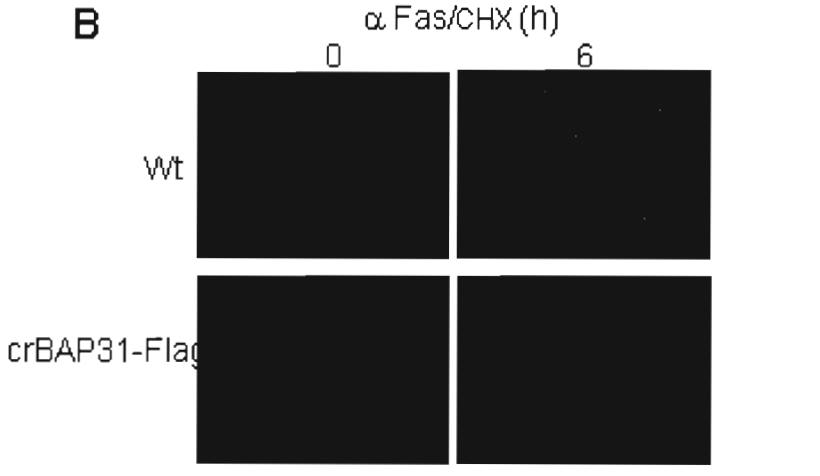
5.5.4 crBAP31 Inhibits Fas-induced Cyt.c Egress from Mitochondria Upstream of Activation of BAX and BAK at the Mitochondrial Outer Membrane

Caspase cleaved BAP31 induces Ca^{2+} transfer between the ER and mitochondria and encourages mitochondrial fission, which sensitizes mitochondria to caspase-8 driven cyt.c release (Breckenridge et al., 2003). Following Fas stimulation, caspase-8 may simultaneously cleave BID and BAP31, activating a tBID-dependent core pathway involving mitochondrial cristae remodeling and BAX/BAK oligomerization (Korsmeyer

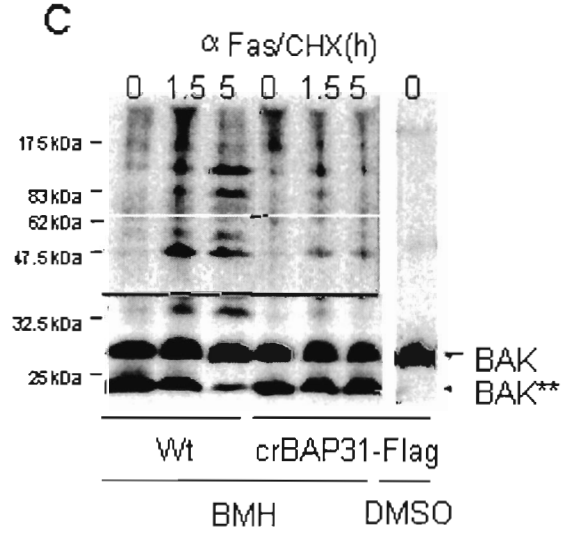
et al., 2000), and a p20-mediated sensitization pathway involving Ca^{2+}-dependent mitochondrial fission (Breckenridge et al., 2003). crBAP31 impairs Fas-induced Ca^{2+} release and, therefore, presumably dismantles the Ca^{2+}-dependent sensitization pathway without effecting the generation or activity of tBID. We examined the effect of crBAP31 on a number of known tBID-driven events following Fas stimulation (Korsmeyer et al., 2000). Figure 5.4A reveals that BID was clipped to tBID following Fas stimulation, and that crBAP31 had little effect on the kinetics or extent of BID cleavage. Similarly, crBAP31 exerted little influence on tBID-driven BAX insertion into the mitochondrial outer membrane. In the absence of Fas ligation, negligible BAX was integrated into mitochondrial membrane as judged by its failure to acquire resistant to alkaline extraction (Time 0, Fig. 5.4A, middle panel). In contrast, Fas stimulation rapidly induced BAX membrane integration in both wt and crBAP31-expressing cells. The inhibition of cyt. c release that is imposed by crBAP31 on the Fas death pathway (Nguyen et al., 2000), therefore, occurs downstream of stimulation-induced insertion of BAX into mitochondrial membrane.

To examine the subsequent steps in the Fas pathway (Korsmeyer et al., 2000), we exploited the observation that BAX undergoes a tBID-dependent conformational change in intact cells, manifesting in the acquisition of reactivity with an antibody directed to the N-terminus of the protein (Desagher et al., 1999). This acquisition of immunoreactivity in intact cells correlates with the appearance of large oligomeric structures containing BAX and the release of cyt.c from mitochondria (Antonsson and Martinou, 2000; Eskes et al., 2000). At a time when significant BAX membrane insertion had occurred (6 h post stimulation of Fas, Fig. 5.4B), a strong Fas-induced fluorescent immunoreactivity was





Immunostaining: α BAX-NT(aa 1-21) (Upstate)



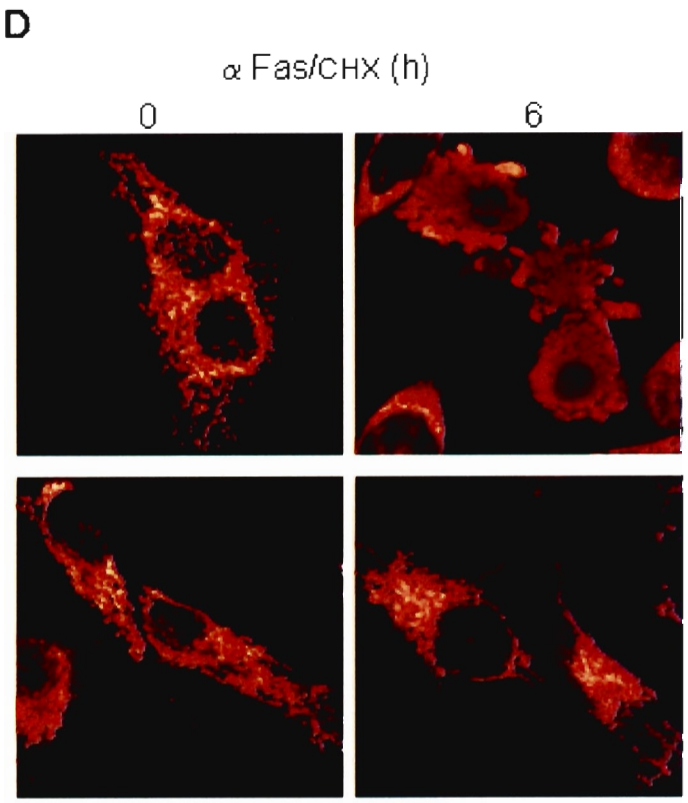


Figure 5.4. KB cells expressing crBAP31 resist Fas-induced apoptotic membrane fragmentation and release of cytochrome c from mitochondria. (A) Wt (parental) KB epithelial cells and KB cells stably expressing crBAP31-Flag were stimulated with 0.5 μg/ml anti (α)-Fas/CHX (see Fig. 1) for the indicated times and the heavy membrane fraction enriched in mitochondria was isolated (26). Aliquots containing equivalent amounts of protein were analyzed by SDS PAGE and immunoblotting either directly (- Alkali) with antibody against BID or after the mitochondrial fraction had been extracted with 0.1 M $NaCO_3^+$ pH 11.5 (+ Alkali) (26), in which case the blots were probed either with antibody against BAX or with antibody against the human mitochondrial outer membrane protein import receptor, TOM20. (C) Parental KB (Wt) cells or KB cells stably expressing crBAP31-Flag were stimulated with anti-Fas as described in (A) for 0 or 6 h, and the cells analyzed by immunofluorescence microscopy employing the conformation-sensitive antibody against BAX, α BAX-NT (aa l-21) (Upstate). (D) As in (B) except that the heavy membrane fraction enriched in mitochondria was isolated after the indicated treatments and incubated with the chemical cross-linking agent BMH or with vehicle (dimethylsulfate [DMSO]) alone. The fractions were then subjected to SDS PAGE and immunoblotted with antibody against BAK. Solid squares indicate cross-linked BAK products and asterisks indicate BAK that harbors an intramolecular cross-link . The positions of protein size markers are also indicated (left). As in A) except mitochondrial morphology and cytochrome c distribution were examined by immunofluorescence microscopy, as described in Materials and Methods. The images shown are typical of multiple independent fields that were examined. Note that in crBAP31 cells cyt. c staining remains mitochondrial and that mitochondrial network has not collapsed, indicating that organelle fission has not occurred. The occurrence of mitochondrial fission in wt cells was confirmed by staining cells with antibody against TOM20 (not shown).

observed for BAX in wt but not in crBAP31-expressing KB cells (Fig. 5.4C). Quantification revealed that greater than 85% of wt cells acquired BAX immunofluorescence following Fas stimulation and that this was reduced to less than 15% for crBAP31 cells. The results of Fig. 5.4B and 5.4C, therefore, indicate that the predicted BAX oligomerization based on immunofluorescence occurs subsequent to BAX membrane insertion and that it is only the oligomerization step that is inhibited by crBAP31. Similarly, a direct analysis of the oligomeric status of BAK, which is constitutively integrated in the mitochondrial outer membrane, in response to Fas stimulation revealed a failure of BAK to enter into higher order structures. Mitochondria isolated from Fas-stimulated wt KB cells and treated with the homobifunctional chemical cross-linker *bis*-maleimidohexane (BMH) (Wei et al., 2000) exhibited a time-dependent shift of BAK into oligomeric structures (Fig. 5.4D), with a concomitant decrease in that fraction of monomeric BAK that can sustain intramolecular cross-linking (Wei et al., 2000) (designated BAK^{**} in Fig. 5.4D). In contrast, the induction of BAK oligomers was strongly curtailed in Fas-stimulated cells expressing crBAP31 (Fig. 5.4D). The inhibitory effect of crBAP31 on BAX and BAK correlated with the reduction in the extent of mitochondria that had undergone large scale fission and cyt.c release in these cells (data not shown; Fig 5.2D; Nguyen et al., 2000). Thus, BAP31 regulated events at the ER that stimulate mitochondrial fission may be important for facilitating complete activation of BAX and BAK and cyt. c release in intact cells during Fas-mediated apoptosis

5.6 Discussion

Initiation of apoptosis following stimulation of the Fas/CD95 family of death receptors involves the recruitment and autoactivation of procaspase-8/a,b at the receptor DISC, resulting in formation of the caspase-8 holoenzyme (Medema et al., 1997). Cleavage of a limited number of substrates by this initiator caspase is then sufficient to commit the cell to die unless downstream pathways are blocked by anti-apoptotic regulators. One set of substrates is the effector procaspases including procaspase-3, at least in certain cellular contexts (Huang et al., 2000; Scaffidi et al., 1998; Scaffidi et al., 1999). Other preferred substrates of caspase-8 that have been identified, however, include RIP (Lin et al., 1999), BID (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998), plectin (Stegh et al., 2000), and BAP31 (Ng et al., 1997). In MCF-7 cells, in which only caspase-8 and not caspases-1,-2,-3,-5,-6,-7,-9, or -10 become strongly activated in response to TNFα stimulation (Janicke et al., 1998; Stegh et al., 2000), we showed that cleavage of both BID and BAP31 correlated with processing of procaspase-8/a,b. Therefore, BID and BAP31 appear to be bonafide caspase-8 targets in vivo. Cleavage of these and other potential caspase-8 substrates downstream of receptor activation may induce critical pro-apoptotic changes at specific cellular sites that collectively support the early events of apoptosis progression in intact cells.

Although the p20 caspase cleavage product of BAP31 itself induces Ca^{2+}-dependent cross-talk with the mitochondria (Breckenridge et al., 2003), we have exploited *Bap31*-null mouse cells to establish that full length BAP31 is in fact a direct inhibitor of apoptosis initiated by caspase-8 in the absence of p20. BAP31 cleavage, therefore, contributes directly to apoptosis progression initiated by this caspase in the Fas death

program by alleviating the antiapoptotic effect of the full length molecule and by generating proapoptotic p20. In Fas stimulated KB cells expressing both crBAP31 and endogenous BAP31, crBAP31 may exert its strong protective effect by maintaining a pool of intact, antiapoptotic BAP31 and by sequestering caspase generated p20.

In KB cells, the expression of crBAP31 impaired the early depletion of ER Ca^{2+} without effecting late elevation of cytosolic Ca^{2+}. Large increases in cytosolic Ca^{2+} are normally associated with capacitative entry of extracellular Ca^{2+} through the plasma membrane, which in some cases can be triggered by depletion of ER Ca^{2+} stores (Putney et al., 2001). The apparent discrepancy between crBAP31 inhibition of ER Ca^{2+} release but not late cytosolic Ca^{2+} increase could be attributed to capacitative Ca^{2+} entry through plasma membrane channels that are activated by Fas signaling irrespective of the ER Ca^{2+} store level, or it may be that crBAP31 inhibition of ER Ca^{2+} release is simply not great enough to prevent subsequent activation of store operated channels in the plasma membrane. Importantly, however, the crBAP31 inhibition of ER Ca^{2+} release did correlate with a reduction in Fas-induced mitochondrial dysfunction, which might be attributed to a block in privileged Ca^{2+} transport between the ER and mitochondria. Indeed, caspase-cleaved BAP31 promotes the transfer of Ca^{2+} between these organelles and stimulates fission of mitochondria independent of BAX or BAK activation (Breckenridge et al., 2003). Interestingly, crBAP31 did not interfere with the apparent cleavage of BID or insertion of BAX into mitochondrial membrane following Fas stimulation. However, the subsequent steps of BAX and BAK oligomerization and release of cyt.c from the organelle were inhibited. This phenomenon could be explained by the "two hit" model for apoptotic mitochondrial transition (Szalai et al., 1999), which postulates that ER Ca^{2+} signals cooperate with a direct BH3-only protein attack on

mitochondria. By dismantling the Ca^{2+} signal transmission from the ER, crBAP31 might prevent mitochondrial fission and other morphological changes, which in turn may be important for the full activation and oligomerization of BAK and BAX in OMM. Thus, cleavage of BAP31 at the ER appears to be required to support these caspase-8- and tBID-initiated steps at mitochondria in intact cells.

5.7 Acknowledgments

We are very grateful to Vishva Dixit, Marta Muzio, and Claudius Vincenz for the gift of MFpk3FLICE and to members of the Shore lab for discussions. D. G. B. is the recipient of a Canadian Institutes of Health doctoral research award. This work was supported by the National Cancer Institute of Canada, the Canadian Institutes of Health Research, the Deutsche Forchungsgemeinschaft through SFB 388, and the Leibniz program.

Chapter 6

General Discussion

6.1 BAP31: a Regulator and Effector of Caspase-8 Pathways

Collectively, the results of this thesis implicate BAP31 as a key regulator and effector of caspase-8, and shed light on the involvement of the ER in diverse apoptotic pathways. In the case of E1A oncogene-induced apoptosis, BAP31 seems to be an integral component of a procaspase-8L activation complex at the ER surface, receiving death signals transmitted from the nucleus and converting them into caspase activation. In cell death pathways in which procaspase-8 is activated by other means, such as death receptor programs, BAP31 functions purely as a caspase-8 substrate. Cleavage of BAP31 converts it from an ER Ca^{2+} release inhibitor to a Ca^{2+} release activator. This switch may help the ER transmit apoptotic Ca^{2+} signals to the mitochondria that activate the fission machinery. Being a regulator and substrate of caspase-8, BAP31 is not likely to play a significant role in cell death pathways that do not utilize this caspase. For example, some death signals induce mitochondrial dysfunction independently of detectable caspase activation. In these cases, BAP31 cleavage would occur downstream of cyt.c release and executioner caspase activation, where it might be of no consequence or, alternatively, function in a feed-back amplification loop to mediate further mitochondrial damage (Green and Reed, 1998).

6.2 BAP31: a Regulator of Procaspase-8L

Like any new finding, the discovery of procaspase-8L leads to more questions than answers. Mechanistically, how is procaspase-8L activated and what exact function do the BAP proteins play? What role does the Nex domain play? How are oncogenic signals transmitted to the ER? How does BCL-2 exert its effect on procaspase-8L

processing? What pathways are activated downstream of procaspase-8L and does procaspase-8L respond to other apoptotic signals? The answers to these questions will hopefully arrive with hard work and carefully experimentation. In the mean time, I propose a number of models that address these questions based on the experimental facts provided within this thesis and a healthy dose of speculation.

6.2.1 Role of the Nex Domain

Aside from the Nex domain, procaspase-8L is identical to procaspase-8/a. However, subcellular fractionation experiments revealed that procaspase-8L is located primarily at the ER, whereas procaspase-8/a and -8/b reside in the cytosol. Therefore, it is likely that Nex domain helps to target procaspase-8L to the ER membrane. Procaspase-8L was sensitive to alkali extraction and trypsin digestion, which are indicative of a peripheral association on the cytosolic face of the ER. It is unlikely that the Nex domain has an intrinsic affinity for membranes because bacterially expressed Nex-GST is recovered in the soluble fraction in the absence of detergent (data not shown). Instead, the Nex domain may constitutively bind a resident ER protein(s), which helps localize procaspase-8L to this membrane. Such proteins are not likely to be BAP31 and BAP29 because procaspase-8L was not observed in the BAP complex prior to E1A signaling and procaspase-8L distribution was not altered in *Bap29,Bap31*-null cells. The identity of procaspase-8L docking proteins at the ER surface might be uncovered through Nex domain protein interaction screens.

However, the Nex domain is not sufficient to allow procaspase-8L targeting to the ER surface. When a Nex domain GFP fusion protein (Nex-GFP) was transfected into cells only a very weak ER staining was observed, with the vast majority of the fusion

protein localized throughout the cytosol (data not shown). Although it cannot be ruled out that GFP masks the ability of Nex to target the ER, these results seem to suggest that another domain within procaspase-8L may contribute in some manner to ER targeting. Procaspase-8 has been reported to have a mitochondrial or membrane distribution in some cells (Stegh et al., 2002; Stegh et al., 2000). A weak intrinsic membrane targeting sequence may exist within procaspase-8 proteins and the combination of the Nex domain and this sequence likely mediates ER targeting of procaspase-8L.

The Nex domain does not seem to directly mediate interaction with BAP proteins. When Nex-EGFP or Nex-HA was transiently co-transfected into H1299 or 293T cells with BAP31-Flag, interactions between the two proteins was not observed (data not shown). Under the same conditions, however, procaspase-8L-HA and BAP31-Flag clearly associate (chapter 2). We favor a model in which the Nex domain helps facilitate constitutive targeting of procaspase-8L to the ER membrane, but that the association between procaspase-8L and the BAP complex is primarily mediated by the DEDs of procaspase-8L. Support for this model comes from transient transfection experiments demonstrating that canonical procaspase-8/a can interact with BAP31-Flag if the two proteins are expressed at very high levels (Ng et al., 1997). Thus, while both isoforms have the potential to associate with the BAP complex, the colocalization of procaspase-8L and BAP proteins at the ER allows these proteins to interact under physiological conditions. The existence of multiple procaspase-8 iosforms permits differential regulation of this important initiator caspase during the various apoptosis programs.

6.2.2 Mechanism of procaspase-8L activation/processing

6.2.2.1 Scaffold model

The fact that *Bap29,31*-double null cells, but not *Bap31*-null or *Bap29*-null cells, resisted E1A-induced procaspase-8L processing and apoptosis, suggests that BAP proteins play redundant roles in mediating the activation of this caspase. The simplest model of how BAP proteins regulate this process is that BAP31/BAP29 homo and/or heteroligomers act as scaffolds for procaspase-8L oligomer assembly and autoactivation. This model stems from the induced proximity model for procaspase-8 autoactivation at the Fas death receptor complex (Medema et al., 1997; Muzio et al., 1996; Muzio et al., 1998). Here, the DED of FADD recruits procaspase-8 to the receptor signaling complex at the plasma membrane (see section 1.6.1). This increases the local concentration of procaspase-8 and reduces its three dimensional movement to two dimensions (Yang et al., 1998), allowing procaspase-8 molecules to oligomerize through homotypic DED interactions. Procaspase-8 has sufficient intrinsic enzymatic activity to mediate trans-cleavage and activation of neighboring procaspase-8 molecules, which triggers a chain reaction of caspase activation. Support for this mechanism is derived from experiments in which autolytic processing of caspase-8 can be stimulated by artificial dimerization in vitro or in vivo (Muzio et al., 1998; Yang et al., 1998).

By a similar mechanism, BAP proteins could concentrate procaspase-8L molecules at certain locations along the ER membrane, facilitating oligomerization and autoactivation of the procaspase. However, procaspase-8L processing was unaffected in *FADD*-null cells suggesting that this process is FADD-independent. The DECC domain of BAP31 shares low sequence similarity with a number of DEDs (Ng et al., 1997) and might directly bind the DEDs in the prodomain of procaspase-8L. DEDs are part of the Death Domain (DD) superfamily, which also includes the DD, CARD and PYRIN families. Despite the low sequence similarity between and within these families (5-25%),

structurally all members form an antiparallel six helical bundle with a greek key topology (Eberstadt et al., 1998; Kadenbach, 1986). However, the mode by which each family forms homotypic interactions varies dramatically. For example corresponding mutations that disrupt the FAS:FADD DD interaction do not effect the DED interaction between FADD and procaspase-8 (Eberstadt et al., 1998). Unfortunately, DED interactions have not been studied extensively and because the DED of FADD is the sole DED structure so far solved, little is known about the interaction surfaces used for DED:DED interactions. Although coiled coil domains normally make homotypic interactions (Burkhard et al., 2001), it is possible that an interface of one or more of the amphipathic α-helices within the coiled coil domain of BAP31 interact directly with the amphipathic α-helices of the procaspase-8L DEDs. Indeed the BAP31 DECC domain contains a basic stretch that aligns with a critical basic stretch found in α-helix 3 of many DEDs (Fig. 6-1). Basic residues are found in this region of BAP29 but the alignment is not optimal. This basic region in α-helix 3 of FADD is critical for its interaction with procaspase-8 (Kaufmann et al., 2002). It would, therefore, be interesting to determine whether mutating this region of the BAP proteins affects their ability to bind procaspase-8L. Of note, however, a conserved phenylalanine in α-helix 2 of FADD that is implicated in the FADD:procaspase-8 interaction (Eberstadt et al., 1998) is not conserved in BAP31 or BAP29, and overall the sequence similarity between BAP proteins and the DED is very low. Therefore, if BAP31 and procaspase-8L do form a direct association, the interaction surface used on the procaspase-8L DED may be somewhat different than that used for the FADD:procaspase-8 interaction.

There is precedence for FADD-independent mechanisms of procaspase-8 activation. For example, procaspase-8 is recruited to the intracellular tail of unligated αvβ3 integrin (Stupack et al., 2001). Integrins are cell adhesion receptors that bind components of the extracellular matrix and transduce survival, proliferation, and migration signals to the cell interior. When certain integrins are not ligated to solid

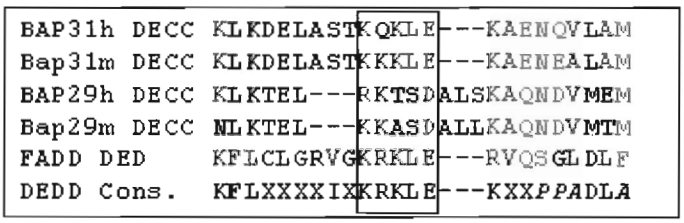


Figure 6.1. Comparison of a conserved basic area (boxed) and surrounding sequence of the DED and the DECC domain. The DEDD consensus sequence was dertemined by aligning this region in FADD, procaspase-8, and procaspase-10. *P*, polar residue; *A*, hydrophobic residue. Red, residues that are strongly conserved within in the consensus sequence; Blue, residues that are loosly defined in the consensus sequence as polar or hydrophopbic. The conserved phenyalanine that is implicated in the FADD/procaspase-8 association is in bold.

support ligands, they oligomerize and transmit an apoptotic signal to the cell by recruiting and activating procaspase-8 in a FADD-independent manner (Stupack et al., 2001). Similarly, cross-linking of the B cell receptor has been linked to proximal FADD-independent procaspase-8 activation, which may also occur at the receptor signaling complex (Besnault et al., 2001). While in each case the mechanism of procaspase-8 activation is unclear, these studies do provide evidence that procaspase-8 is capable of forming pleiotropic interactions with different signaling complexes. Furthermore, it is interesting that in each case the activation of procaspase-8 is associated with the oligomerization of a transmembrane protein, which presumably induces clustering and autoactivation of the procaspase. In this regard, it is noteworthy that BAP31 oligomers

have been observed both in vivo (Ng, 1998) and in vitro (see below), which is often a general feature of the coiled coil containing proteins (Burkhard et al., 2001).

To determine whether BAP31 and procaspase-8L could directly associate, I developed an in vitro binding assay utilizing recombinant bacterially expressed DECC domain of BAP31 (GST-DECC) and in vitro translated procaspase-8L. Using this system I did not observe a specific interaction between the two proteins (data not shown). This result could be explained by the inability of either protein to mature to its proper folding conformation during in vitro expression. Therefore, a positive control was included in which in vitro translated DECC was incubated with recombinant GST-DECC. Despite the fact that the DECC domain can homodimerize in vivo (Ng, 1998; Ng and Shore, 1998), no interaction was observed in vitro. It is likely that the coiled coil domain of BAP31 aggregates into a nonproductive oligomer in vitro. In fact, GST-DECC purified as an isochromatic dodecamer following FPLC chromatography (M. Nguyen and G. Shore, unpublished data). Therefore, determining whether or not the DECC domain and procaspase-8L directly interact must await optimization of the GST-DECC purification protocol.

BAP proteins might interact with procaspase-8L via an adapter molecule. Overexpression of a CED-4 DN mutant blocked the association observed between BAP31-Flag and procaspase-8 in cotransfected 293T cells (Ng and Shore, 1998), suggesting that the CED-4 DN mutant might prevent an endogenous adapter protein from mediation the BAP31/procaspase-8 interaction. As stated above, if BAP proteins do recruit an adapter it is unlikely to be FADD. Similarly, APAF-1, which has also been reported to bind procaspase-8 (Hu et al., 1998), was not present in the immunoisolated crBAP31 complex (data not shown). BAP31 might interact with a coiled coil containing

protein that in turn recruits procaspase-8L to the complex. Immunoisolation of BAP31-Flag from stable expressing cells followed by analysis of the native complex by size exclusion chromatography revealed that BAP31 is part of an approximately 700 -1000 kDa complex in healthy cells (Boyer, 1999). Given the size of the native BAP31-Flag complex, it is possible that a BAP associated protein at the ER mediates the interaction with procaspase-8L. In this scenario, BAP proteins might simply act as structural components that stabilize the complex or help transmit the E1A signal to the associated protein, which inturn recruits and activates procaspase-8L. Further proteomic analysis of the immunoisolated complex from E1A stimulated cells might provide insight into this issue.

6.2.2.2 Recruitment of an upstream protease

BAP proteins could also recruit or regulate another protease that clips off the prodomain of procaspase-8L, releasing the enzymatic subunits to the cytosol. This mechanism stems from studies on procaspase-12, which is also localized at cytosolic face of the ER (see section 1.8.1.2). Nakagawa and Yuan have found that following ER stress procaspase-12 undergoes m-calpain-dependent proteolysis within its prodomain, which releases the active enzymatic subunits of the caspase (Nakagawa and Yuan, 2000). Because the proteolytic activity of calpains is dependent on Ca^{2+}, calpains might be stimulated by local Ca^{2+} transients near the ER membrane under conditions of ER stress. By analogy, it is possible that procaspase-8L could also be cleaved by a calpain at the ER. This raises the question of whether the ability of BAP31 to control ER Ca^{2+} homeostasis might somehow be related to its ability to regulate procaspase-8L activation. For example, BAP31 might recruit procaspase-8L to an area of privileged Ca^{2+} signaling,

causing procaspase-8L proteolysis by calpain. Alternatively, procaspase-8L might be cleaved by another protease residing at the ER membrane in a BAP-dependent manner. These models seem to disagree with the finding that zVAD-fmk, a caspase-specific inhibitor, blocked procaspase-8L processing. However, it cannot be ruled out that procaspase-8L undergoes a very limited and undetectable initial caspase-independent cleavage, activating a small pool of caspase-8, which then cleaves the remaining pool of procaspase-8L. Insight into this type of mechanism could be gained by examining the effect of various protease inhibitors on E1A-induced cleavage of procaspase-8L in vivo and by determining the exact cleavage site within the prodomain.

We also cannot rule out the possibility that an upstream caspase cleaves procaspase-8L at the BAP complex. ER localized caspase-12 is an unlikely candidate because it is activated only under conditions of ER stress (Nakagawa et al., 2000), whereas procaspase-8L processing is observed in response to most apoptotic stimuli (data not shown). Given that E1A signals also induce the expression of several BH3-only proteins that stimulate cyt.c release (Mathai et al., 2002; Vogelstein et al., 2000), it could be argued that BAP-dependent procaspase-8L processing is mediated by a caspase activated downstream of the apoptosome. However, expression of a procaspase-9 DN mutant, which prevents cyt.c-dependent caspase activation (Srinivasula et al., 1998), did not effect E1A induced procaspase-8L processing (data not shown). This result suggests that in the E1A pathway procaspase-8L processing occurs upstream or in parallel to mitochondrial damage and apoptosome-dependent caspase activation. Nonetheless, another unidentified caspase could forseeably cleave procaspase-8L at the ER

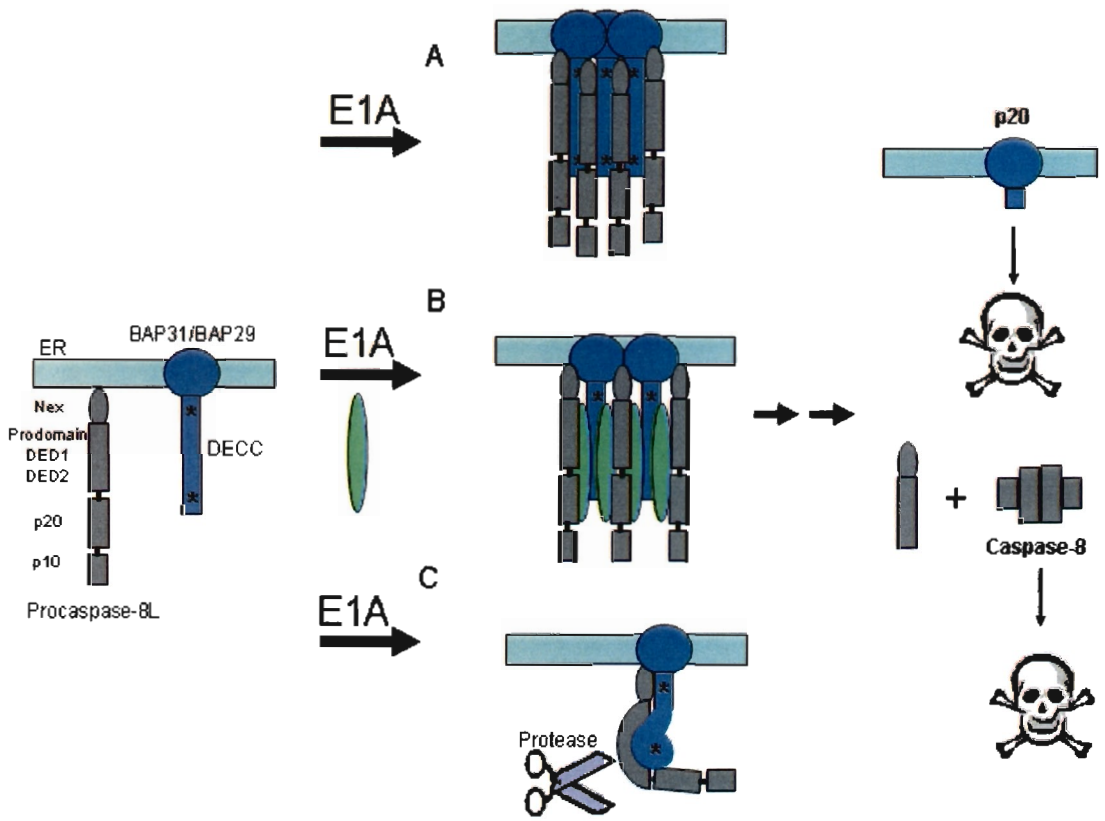


Figure 6.2. Models of BAP-regulated procaspase-8L activation. A) Scaffold model; B) BAP recruitment of an adapter molecule; C) chaperone assisted cleavage or recruitment of an upstream protease. See text for details. Activation yields the prodomain fragment (containing the Nex domain) and mature caspase-8, which is expected to cleave BAP31 and generate p20.

6.2.2.3 Chaperone assisted proteolysis

A number of heat shock proteins (HSPs), have been implicated in binding and regulating the activation of caspase-9 and -3 (Beere, 2001). HSPs are stress-inducible chaperonins that normally function in protein folding, polypeptide subunit assembly, and translocation of proteins across membranes (Bukau and Horwich, 1998). During apoptosis, HSP10 and HSP60 bind procaspase-3 and convert it into a protease-sensitive

state, which enhances its cleavage by other caspases (Samali et al., 1999; Xanthoudakis et al., 1999). Since BAP31 has been implicated as an ER export chaperone itself (Annaert et al., 1997; Spiliotis et al., 2000), it is possible BAP proteins assist procaspase-8L achieve a structural conformation that is required for self-activation or for recognition by an upstream caspase.

6.2.3 E1A Signaling to the BAP Complex

The E1A region of human adenovirus type 5 (Ad5) encodes two differentially spliced 12 S and 13 S mRNAs that give rise to 243 and 289 residue (243R, 289R) proteins, respectively (reviewed in Bayley, 1994). Both of the E1A proteins promote cell cycle progression and unscheduled DNA synthesis in terminally differentiated epithelial cells, the normal cellular targets of Ad5. Both products are transcription factors that exert their effects through two domains within the molecules called conserved region 1 and 2 (CR1 and CR2) in addition to a third domain near the amino terminus. 289R E1A contains an additional domain, CR3, that enables it to activate other early viral units. The E1A proteins promote cell growth by binding and inactivating members of the RB family of tumor suppressors, which releases E2F transcription factors to promote S-phase gene expression, and by association with the p300/CBP family of histone acetylases (Bayley, 1994; Shikama et al., 1999). The combined effects of these interactions results in two opposing signals: a growth promoting signal leading to cell cycle progression and unscheduled DNA synthesis, and a counteracting signal leading to the accumulation of the p53 tumor suppressor and apoptosis. E1A's apoptosis inducing activity is thought to be a result of its transforming potential because mutations that disrupt E1A's ability to promote cell cycle progression also abolish its ability to induce apoptosis. The early

region 1B (E1B) of Ad5 encodes two viral inhibitors of apoptosis, which allow E1A to promote interrupted DNA synthesis that is necessary for viral replication. E1A can transform cells when coexpressed with E1B because its oncogenic signals are uncoupled from apoptosis (Branton et al., 1985). Therefore, the study of E1A proteins in the absence of E1B and other viral products represents an excellent model of oncogene-induced apoptosis. In chapter 2, this was achieved by infecting KB cells with an Ad5 viral vector that was engineered to deliver the 243R product of E1A as the only viral product (Debbas and White, 1993; Lowe and Ruley, 1993).

E1A signals are transmitted to p53 through the tumor suppressor $p19^{ARF}$ (Lowe, 1999). Expression of E1A increases the expression of $p19^{ARF}$, which then complexes with p53 and inhibits MDM2, a protein ubiquitin ligase that mediates p53 degradation. This leads to an induction of p53 levels and activity resulting in p53- dependent transactivation of an ever increasing number of apoptotic genes (Ljungman, 2000). These genes encode proapoptotic members of the Bcl-2 family, including the BH3-only proteins BID, BIK, PUMA, and NOXA, and multidomain BAX; other apoptotic proteins with known functions, such as TRAIL-R2 and Fas; and proapoptotic proteins with unknown functions such as PERP, PIDD, and Scotin (Bourdon et al., 2002; Lin et al., 2000; Ljungman, 2000; Lowe, 1999). The combined actions of these proteins is thought to stimulate multiple, and in some cases redundant, cell death pathways, ensuring that cells that have undergone oncogenic stress or extensive DNA damage are efficiently destroyed. Interestingly, a number of these proteins, including BIK, NOXA, BAX, PIDD, and Scotin, either completely or partially localize at the ER following activation of p53 (Bourdon et al., 2002; Lin et al., 2000; Mathai et al., 2002; Oda et al., 2000). These proteins are, therefore, candidate regulators of the BAP/procaspase-8L complex.

One might envision several mechanisms by which these proteins mediate BAP-dependent procapase-8L activation. One possibility is that BH3-only BIK regulates the BAP complex in the same way EGL-1 regulates caspase activation in *C. elegans*. As discussed in section 1.3, EGL-1 binds CED-9, relieving CED-9 inhibtion of CED-4, and CED-4 then activates CED-3 (Metzstein et al., 1998). BCL-2 can compensate for a *ced-9* loss-of-function mutation in nematodes (Hengartner and Horvitz, 1994) indicating that BCL-2 may also directly regulate caspase activation complexes in mammalian cells. BIK binds BCL-2 at the ER (M.Germain and G. Shore, unpublished) and BCL-2 binds BAP31 (Ng et al., 1997; Ng and Shore, 1998) and inhibits procaspase-8L processing (Figure 2.4). Following oncogenic stress, BIK might relieve the inhibitory effect of BCL-2 on procaspase-8L processing by binding BCL-2 and releasing BAP31. BAP31 might then oligomerize and activate procaspase-8L. Consistent with this model, overexpression of BIK induces procaspase-8L processing. Furthermore, BIK-induced procaspase-8L processing can be blocked by overexpressing of b5-BCL-2 (i.e. ER localized BCL-2) (S. Beuler, M. Germain and G. Shore; unpublished). It will be important to determine whether E1A-induced procaspase-8L processing is inhibited when BIK expression is abolished by RNAi knockdown, or in mice deficient for the BIK homolog BLK. Since NOXA can partially localize at the ER, investigation into its ability to influence procaspase-8L processing is also warranted.

Alternatively, other ER localized proteins that are upregulated by p53 could signal to the BAP complex. For example, PIDD or Scotin might bind the BAP proteins and induce their oligomerization and recruitment of procaspase-8L, or these proteins could be adapter molecules that facilitate the interaction between procaspase-8L and BAP31 and BAP29. On the other hand, BAX might somehow regulate the BAP complex even

though it does not directly associate with BAP31 (Ng et al., 1997). The role of multidomain proapoptotics like BAX and BAK at the ER is not yet clear. These proteins have been implicated in regulating ER Ca^{2+} release, which could inturn activate a protease that cleaves procaspase-8L at the BAP complex or induce a conformational change in BAP proteins that leads to procaspase-8L recruitment and activation.

6.2.4 BCL-2 Regulation of BAP-dependent Procaspase-8L Processing

There are several other mechanisms by which excess BCL-2 might regulate the BAP/Procaspase-8L complex. For example, BCL-2 could sequester a co-factor that is crucial for procaspase-8L activation, inhibit the activation of an upstream protease, or influence ER Ca^{2+} homeostasis in a manner that disrupts procaspase-8L processing. All of these models are consistent with the observation that BCL-2 prevented E1A induced processing of procaspase-8L, but not the recruitment of procaspase-8L to the BAP complex.

6.2.5 Procaspase-8L Signaling Cascades.

E1A-induced procaspase-8L processing yielded an approximately 35 kDa N-terminal fragment that was detected by the anti-Nex domain antibody. This fragment is presumably the full prodomain (Nex domain and both DEDs, expected size 33 kDa). If procaspase-8L undergoes autocatalytic activation at the BAP complex in a manner similar to procaspase-8 activation at death receptors, the first cleavage would occur between the p10 and p18 subunits (Medema et al., 1997). The p18 and p10 subunits likely remain noncovalently associated and partially assemble with neighboring subunits into the heterotetrameric holoenyzme before cleavage occurs between the p18 subunit and the

prodomain. After this cleavage step, the mature p18/p10 heterotetramer could be released to the cytosol. If procaspase-8L activation is mediated by an upstream protease, on the other hand, then the protease would likely clip procaspase-8L between the prodomain and the p18 subunit. This might release partially assembled p18/p10 complexes to the cytosol, the active enzyme being generated after self-cleavage between the subunits. In either case, the fact that no processing intermediates immunoprecipitated with crBAP31-Flag suggests that these processing steps are fast and that all cleavage fragments rapidly dissociate from the BAP complex.

Regardless of the mechanism of activation, mature caspase-8 that is derived from procaspase-8L is likely indistinguishable from caspase-8 that is derived from procaspase-8 activation at death receptors. Thus, activated procaspase-8L likely targets many of the same substrates that are cleaved by caspase-8 following death receptor stimulation. These substrates include procaspase-8 itself, the BH3-only protein BID, the cytolinker plectin, BAP31, and executioner procaspase-3. Cleavage of cytosolic procaspase-8 may amplify weak caspase-8 signals (Scaffidi et al., 1998; Stegh et al., 2002; Stegh et al., 2000), which may be particularly relevant in the E1A pathway because the expression of procaspase-8L is very low. E1A-induced processing of procaspase-8/a and -8/b was inhibited in *Bap29,Bap31*-null cells (Figure 2.7C) suggesting that mature caspase-8 released from the ER does process cytosolic procaspase-8/a and -8/b. Similarly, caspase-8 (derived from procaspase-8L) cleavage of BAP31 and BID, in cooperation with other BH3-only proteins upregulated by E1A (Mathai et al., 2002; Vogelstein et al., 2000), might trigger mitochondrial damage and further increase caspase activation. Because BAP31 is located at the site of procaspase-8L activation, it may be a privileged procaspase-8L target that is cleaved before other substrates.

Future experiments should reveal whether procaspase-8L plays an important role in other cell death pathways. Clearly DNA stress pathways that activate p53 (Ljungman, 2000) would also be predicted to stimulate BAP-dependent procaspase-8L processing at the ER. Accordingly, we observed that overexpression of p53 could bypass the requirement for E1A and stimulate recruitment of procaspase-8L to crBAP31-Flag and procaspase-8L processing (data not shown). Given its location at the ER, procaspase-8L might also be expected to function in ER stress induced apoptosis. Indeed, I observed procaspase-8L processing following insult with TN or TG, but *Bap31,29*-double null cells did not prove resistant to these ER stress agents, suggesting that procaspase-8L processing occurred independent of BAP proteins (data not shown). One possibility is that caspase-12 or downstream executioner caspases cleave procaspase-8L in this pathway. Selective knockdown of procaspase-8L expression with RNAi should help determine whether procaspase-8L initiates caspase activation in response to apoptotic signals other than oncogenic stress.

6.3 BAP31: a Caspase-8 Substrate that Regulates Ca^{2+}-Dependent Proapoptotic Crosstalk between the ER and Mitochondria.

6.3.1 Regulation of Fas-induced Apoptosis by BAP31

In vitro, BAP31 is preferentially cleaved by caspase-8 (Ng et al., 1997), and the results of chapter 5 suggest that this is also the case in vivo. Thus, in TNF-α-stimulated MCF-7 cells, in which caspase-8 is the only caspase to be significantly activated (Stegh et al., 2000), BAP31 cleavage occurs rapidly and with similar kinetics as BID cleavage. It is not surprising, therefore, that crBAP31 exerts strong protection against Fas-induced apoptosis, where caspase-8 plays an essential and nonredundant role at the apex of the

pathway. In crBAP31 cells the activation of caspase-8 and the cleavage of caspase-8 substrates, such as BID and endogenous BAP31, were uneffected. However, typical morphological features of apoptosis, including plasma membrane blebbing, cell rounding and detachment, actin depolymerization, and nuclear membrane disassembly were strongly impaired by crBAP31. Despite the generation tBID, and tBID-dependent insertion of BAX into the OMM, mitochondria maintained $\Delta\Psi_m$ and resisted the release of cyt.c to cytosol. The activation of downstream caspase-3 and cleavage of caspase-3 substrates, such as PARP, in contrast, were only partially inhibited by crBAP31. This likely reflects the ability of crBAP31 to inhibit cyt.c-dependent caspase activation through the apoptosome, but not direct proteolysis and activation of procaspase-3 by caspase-8. By shutting down the mitochondrial pathway to caspase-3 activation, but not the caspase-8 pathway, crBAP31 only partially blocks downstream caspase activation. Interestingly however, some caspase-dependent cell disassembly processes, such as DNA degradation and loss of phosphatidylserine asymmetry at the plasma membrane, were unaffected by crBAP31. This observation argues that the partial inhibition of caspase-3 activity observed in crBAP31 cells cannot explain its strong protective effect on mitochondrial dysfunction and cytoplasmic apoptosis. In contrast, this protective effect is more likely to pertain to the ability of crBAP31 to directly regulate an ER based apoptotic pathway.

The fact that crBAP31 inhibited diverse features of apoptosis suggests that it exerts an effect on a core process that controls the onset of several downstream cell death processes. The actinomyosin network is known to regulate apoptotic cell rounding and detachment, membrane blebbing, cell condensation (Mills et al., 1999). It is conceivable that by maintaining an interaction with actinomyosin, crBAP31 is able to halt these

processes. Given the vast size and diversity of the actinomyosin network, however, it is unclear how binding to one ER protein could prevent the various actin and myosin-dependent processes required during the execution phase. Furthermore, the interactions between endogenous BAP31 and γ-Actin and crBAP31 and γ-Actin are lost during Fas-mediated apoptosis (Figure 3.12; and data not shown). Therefore, crBAP31 does not appear to exert its antiapoptotic effect by maintaining contact with actin and myosin. Nevertheless, this association may be relevant to BAP31's potential role in ER export (Annaert et al., 1997; Spiliotis et al., 2000).

The discoveries in chapter 4 and 5 that crBAP31 inhibits Fas-induced release of Ca^{2+} from the ER, and that Ca^{2+} release is required for p20's proapoptotic function, support the idea that crBAP31's pleiotropic affects are mediated by its influence ER Ca^{2+} signaling. Several studies have established that Ca^{2+} transmission between the ER and mitochondria can control mitochondrial cyt.c release (reviewed in Breckenridge and Shore, 2002; Ferrari et al., 2002; Hajnoczky et al., 2000a). We believe that by preventing Ca^{2+}-dependent ER-mitochondrial apoptotic cross-talk (and the ensuing fission of mitochondria, mitochondrial release of proapoptotic factors, and loss of respiratory function) crBAP31 indirectly prevents cytoplasmic apoptosis and allows Fas-stimulated cells to remain viable (Figure 3.7). Mitochondria may release factors required for cytoplasmic apoptosis, or indirectly regulate these processes by altering cytosolic pH or ion fluxes (Matsuyama et al., 2000), or by activating a required caspase. This conclusion is supported by the fact that overexpression of BCL-2, which also blocks mitochondrial fission, cyt.c release, and loss of $\Delta\Psi_m$, blocks Fas-mediated cytoplasmic apoptosis in KB cells (data not shown).

6.3.2 Regulation of ER Ca^{2+} Release by BAP31 and p20

The simplest explanation for the observed effect of crBAP31 and p20 on ER Ca^{2+} release is that caspase cleavage of BAP31 converts it from Ca^{2+} release inhibitor to a Ca^{2+} release activator. BAP31/p20 could conceivably modulate ER Ca^{2+} release in four ways: 1) by forming a Ca^{2+} channel itself or in cooperation with other ER membrane proteins; 2) by influencing a preexisting Ca^{2+} channel of the ER membrane; 3) by regulating Ca^{2+} leak form the ER; or 4) by influencing Ca^{2+} uptake by the ER.

Our original observation that crBAP31-inhibited Fas-induced apoptosis in KB cells could, on the one hand, be explained by the ability of crBAP31 to bind and inactivate endogenous p20. Adp20-induced apoptosis is strongly inhibited in cells overexpessing crBAP31-Flag or BAP31-Flag, suggesting that full-length BAP31 can sequester p20 in an inactive state (data not shown). On the other hand, by preserving a pool of intact BAP31, crBAP31 could negatively regulate apoptosis by some other means. We recently published an article documenting that reconstitution of crBAP31 into Bap31-null cells conferred resistance to caspase-8-initiated events (Wang et al., 2003 and Chapter 5). Our conclusion from these experiments was that crBAP31 could inhibit apoptosis independent of p20. A natural extension of this declaration is that p20 and BAP31 do not directly modulate Ca^{2+} passage through the ER membrane. If caspase cleavage of BAP31 were to convert if from a closed channel to open channel, for example, cells deficient in Bap31 would lack the release channel and be expected to resist caspase-8 signaling and reconstitution of crBAP31 in these cells would have no added effect. The only remaining hypothesis is that during apoptosis crBAP31 and p20 independently exert their effect on a third party.

Unfortunately, this may be a naïve explanation. If BAP31 is a regulator of physiological ER Ca^{2+} release and/or homeostasis, loss of BAP31 might alter (perhaps increase) the steady state $[Ca^{2+}]_{ER}$. This change in ER Ca^{2+} content could then affect the sensitivity of the cells to apoptosis (Pinton et al., 2001). Take, for example, calreticulin, a major Ca^{2+} binding protein and chaperone of the ER lumen. Calreticulin controls ER Ca^{2+} stores by regulating IP_3R and SERCA activity and by sequestering free Ca^{2+} in the lumen (Corbett and Michalak, 2000). Cells overexpressing calreticulin have a higher ER Ca^{2+} content and are sensitized to apoptosis, whereas cells deficient in calreticulin have a decreased ER Ca^{2+} content and resist apoptosis (Nakamura et al., 2000). BCL-2, BCL-xL, BAX and BAK provide other examples. BCL-2 overexpression lowers ER Ca^{2+} content and inhibits apoptosis, and recent evidence suggests that BCL-2 knockdown by siRNA increases ER content and sensitizes cells to apoptosis (Oakes, 2003). Conversly, cells deficient in BAK and BAX have a decreased ER Ca^{2+} store and resist apoptosis (Oakes, 2003). Overexpression of BCL-xL decreases the expression of the IP_3R and might alter the ER Ca^{2+} load (Li et al., 2002a). The bottom line is that manipulating the expression of genes that regulate ER Ca^{2+} homeostasis can alter the ER store and indirectly affect the sensitivity of a cell to apoptotic stimuli. Given that BAP31-independent Ca^{2+} release pathways may also play a role in caspase-8 induced cyt.c release (the IP3R, for example, has been implicated in Fas signaling (Jayaraman and Marks, 1997)) than an elevation in $[Ca^{2+}]_{ER}$ in *Bap31*-null cells might in fact increase the release of Ca^{2+} from these channels. Reconstitution of crBAP31 at levels 2-3 fold above normal wt levels might then decrease the $[Ca^{2+}]_{ER}$ below normal levels and, inturn, protect cells against caspase-8 induced cyt.c release. Clearly, the results obtained in the *Bap31*-null

cells must not be overinterpreted until the ER Ca^{2+} content of these cells is carefully assessed.

Here in the comfort of a Ph.D. thesis (free of a scrutinous peer review process), for the benefit of the discussion I will not form a bias based on the aforementioned results of crBAP31 reconstitution in *Bap31*-null cells. With that in mind, I discuss the four models that could account for how BAP31 cleavage influences ER Ca^{2+} release during Fas-mediated apoptosis.

6.3.2.1 BAP31: a Putative Ca^{2+} Channel ?

The crystal structure of the *Streptomyces lividans* potassium channel (KcsA) (Doyle et al., 1998) has provided an understanding of the minimum components necessary for ion conduction through a membrane. KcsA, like all ion channels, is a tetramer. Each monomer contains two TM helixes that are separated by an extracellular loop containing a hydrophobic helix (pore helix) and a 5 amino acid ion selectivity filter that is highly conserved in other ion channels (Catterall, 1995). The four pairs of helices form an inverted teepee, the wide base is extracellular and cradles the four pore helixes and selectivity filters, which run inwards towards the pore axis (Armstrong, 1998). The backbone carbonyls of the four selectivity filters coordinate the ion, and ion selection is thought to be determined by the position of the filters relative to the pore. Thus, the minimum requirement for an ion selective channel subunit appears to be two transmembrane helixes, a pore helix and a selectivity filter.

Examination of voltage-gated Ca^{2+} channels at the plasma membrane and the IP_3R and RyR Ca^{2+} channels at the ER, revealed that each contains these three basic requirements of an ion selective channel. The pore forming subunit of voltage-gated Ca^{2+}

channels is the α1 subunit. Instead of forming a tetramer, α1 is a single polypeptide with four repeated domains, each containing 6 TM helixes (Catterall, 1995). Both the IP_3R and RyR Ca^{2+} release channels are tetramers, the monomers of each are structurally similar - a long N-terminal cytosolic regulatory region followed by 6 or 4 TM domains, respectively, and a short cytosolic N-terminal domain (Patel et al., 1999). The pore helix and selectivity filter is present in the lumenal loop between the last two TM helixes of the IP_3R and the RyR and in the last two TM helixes of each domain of the α1 subunit of voltage gated channels (Catterall, 1995; Shah and Sowdhamini, 2001; Williams, 2002). Mutagenesis and three dimensional structure analysis of all these receptors has revealed that these two TM helixes and their pore helix and selectivity filters form the pore of each channel (Catterall, 1995; Jiang et al., 2002; Shah and Sowdhamini, 2001; Stokes and Wagenknecht, 2000). In the case of the IP_3R, it has even been demonstrated that this region alone is sufficient for multimerization and ion conductance (Galvan et al., 1999). Therefore, the minimal structural requirements for a Ca^{2+} selective channel also seems to be four sets of transmembrane helixes, each containing a pore helix and selectivity filter. Of note, the cytosolic domains of each these channels are responsible for gating the pore when the channel is in the inactive state.

Given that each of BAP31's TM domains can form transmembrane helixes (Figure 6.3A), and homooligomerize in the ER membrane (Ng and Shore, 1998), it is conceivable that BAP31 could form a crude tetrameric Ca^{2+} channel. The cytosolic coiled coil domain of each monomer might wrap around one another, forming a supercoil (Burkhard et al., 2001) that gates the pore. During apoptosis, caspase cleavage of BAP31 removes the coiled coil domain and could permit Ca^{2+} release through the pore. If

positioned at sites where the ER is juxtaposed to mitochondria, p20 might only have to allow a nonselective permeation of Ca^{2+} across the membrane. The large Ca^{2+} concentration gradient across the ER membrane would cause a flux of Ca^{2+} into the cytosolic junction between the ER/mitochondrial membranes sufficient to stimulate the IMM Ca^{2+} uniporter. Importantly, BAP31 is enriched in the smooth ER (Ng, 1998), a region where Ca^{2+} signaling hotspots and mitochondrial contact sites are observed (Berridge, 2002; Hajnoczky et al., 2000b).

Inspection of the lumenal domain between TM2 and TM3 of BAP31 did not reveal an obvious pore helix or selectivity filter. However, if p20 were to simply mediate Ca^{2+} leak from the ER during apoptosis, these elements would not be required. The molecular complexity of the voltage gated channels and the IP_3R and RyR can be attributed to their important roles in stimulating ion specific signals. For example, the voltage-gated channels must respond to cell depolarization with a burst of Ca^{2+} influx (Catterall, 1995). Therefore, the $\alpha 1$ subunit is equipped with a number of regulatory domains and subunits that allow precise, rapid, and voltage-dependent channel activation and inactivation. Likewise, the RyR and IP_3R are responsible for generating a wide diversity of multifaceted Ca^{2+} signals, including Ca^{2+} waves that vary in spatiotemporal pattern, frequency, and amplitude (Berridge et al., 1998). To achieve this, the ER channels are subject to modulation by a variety of inputs, including second messengers, local ATP concentration, protein phosphorylation, interacting proteins, and biphasic regulation by cytosolic and lumenal Ca^{2+} (Patel et al., 1999). In contrast, if p20 is a channel that only functions during apoptosis it would not require such accessories. This reasoning is justified by comparison to the multidomain Bcl-2 proteins, which structurally

resemble the TM regions of diphtheria toxin (Muchmore et al., 1996) and seem to form simple oligomeric membrane pores in the OMM (Korsmeyer et al., 2000).

p20-induced Ca^{2+} release occurred over 5-10 h, which seems slow for a Ca^{2+} channel. However, because the evaluation of ER and mitochondrial Ca^{2+} stores was measured from whole cell populations, it is impossible to determine whether the observed kinetics of Ca^{2+} release actually reflect a slow release or asynchronicity between cells. Rapid but asynchrotic release of ER Ca^{2+} would still appear as a gradual release over time when measured in a cell population. Analyzing p20- and Fas-induced Ca^{2+} release from the ER at the single cell level might clarify this issue. In addition, it should be noted that the Ca^{2+} release that occurs over the course of Adp20 infection does not necessarily reflect p20-induced Ca^{2+} release during apoptosis. In a physiological setting cleavage of BAP31 may be coincident with the cleavage of ER substrates that further regulate ER Ca^{2+} release.

6.3.2.2 Regulation of a Ca^{2+} Channel by BAP31

BAP31 could also be a modulatory subunit of an ER Ca^{2+} release channel. Using a split-ubiquitin yeast 2-hybrid assay that was modified to detect interactions between integral membrane proteins (Stagljar et al., 1998), we recently identified the putative ion channel protein A4 as a BAP31 binding partner (Wang et al., 2003). A4 is a widely expressed, 17 kDa protein that spans the ER membrane 4 times (Breitwieser et al., 1997). Endogenous A4 interacts with both endogenous BAP31 and p20 (Wang et al., 2003). Like BAP31, A4 is able to multimerize and has TM helices containing both acidic and basic residues (Breitwieser et al., 1997). These charged residues may form salt bridges

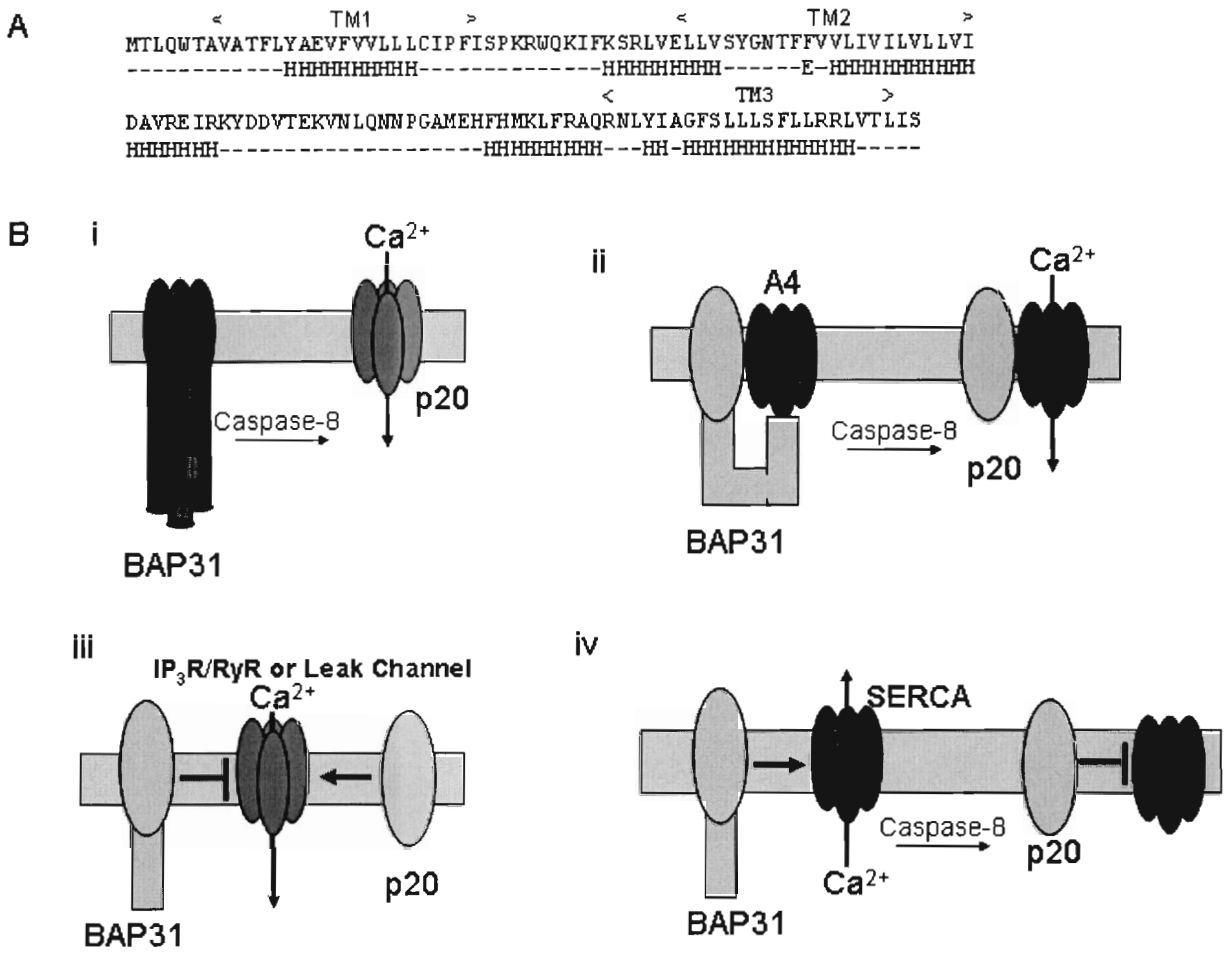


Figure 6.3. A) Secondary structure prediction of the TM regions of BAP31. H, helix. B) Models of BAP31-regulated Ca^{2+} release for the ER. i) BAP31 is a Ca^{2+} channel itself that is opened upon cleavage by caspase-8; ii) BAP31 and p20 regulate the A4 channel; iii) BAP31 and p20 regulate the IP3R, RyR, or Ca^{2+} leak channels; iv) BAP31 and p20 influence Ca^{2+} influx into the ER.

that stabilize an intermembrane interaction between BAP31 and A4. Breitwiesser et al., observed a novel 28 Ps conductance channel in isolated nuclei from *Xenopus laevis* oocytes overexpressing A4, and proposed that A4 possesses ion conductance properties (Breitwieser et al., 1997). If A4 is indeed a Ca^{2+} channel, full length BAP31 might promote the closed state of the pore and caspase cleavage of BAP31 and generation of p20 might induce a conformation change in the complex that opens the pore. On the

other hand, since A4 does not have a substantial cytosolic region the cytosolic tail of BAP31 might play a role in gating the pore. In this case, caspase-8 cleavage of BAP31 may remove the gate and open the channel during apoptosis.

Although a link between BAP31 and the IP_3R or RyR has never been established, it is possible that BAP31 might somehow, directly or indirectly, regulate Ca^{2+} release through these channels during apoptosis. Both the IP_3R and RyR have been implicated as important in certain apoptosis pathways (Hajnoczky et al., 2000a; Jayaraman and Marks, 1997) and, therefore, investigation into a possible BAP31 regulatory role is warranted. BAP31 or p20 could either bind one of the receptors or control a stress signals that modulates receptor phosphorylation or interaction with a regulatory protein. p20-induced Ca^{2+} release from the ER was not significantly affected when cells were preincubated with dantrolene, a RyR inhibitor, or Xestospongin C, a specific IP_3R inhibitor (data not shown). In addition, crBAP31 had no effect on agonist-induced Ca^{2+} release from the ER (M. Michalak, personal communication). These results may suggest that the IP_3R and RyR are not targets of BAP31 regulation.

6.3.2.3 BAP31 Regulation of Ca^{2+} Leak Channels.

The steady state level of ER Ca^{2+} is a balance between Ca^{2+} influx through SERCA pumps and Ca^{2+} leak out of the organelle. Ca^{2+} leak from the ER is a poorly understood process (Camello et al., 2002). Possible Ca^{2+} escape routes include back flux through SERCA pumps, the polypeptide translocon, the transporter associated with antigen processing (TAP) complex, and BCL-2, BAX and BAK pores (Camello et al., 2002). Since BAP31 is known to associate BCL-2, and it has been proposed that BAP31 associates with the TAP/MHC I complex (Spiliotis et al., 2000), it is possible that BAP31

and p20 might influence Ca^{2+} leakage through one of these pores during apoptosis. Also possible, is that BAP31 regulates BAX/BAK pores in the ER membrane (Nutt et al., 2002), although BAP31 does not appear to directly interact with these proteins (Ng et al., 1997). Again, if these pores were positioned at sites proximal to mitochondria, the Ca^{2+} concentration gradient across the ER would be sufficient to permit transport of Ca^{2+} between the two organelles.

6.3.3.4 BAP31 Regulates Ca^{2+} Influx into the ER

Similarly, BAP31 cleavage could influence the rate of Ca^{2+} entry into the ER during apoptosis. If SERCA activity was inhibited, the ER content would decrease and backward leakage through SERCA might drive Ca^{2+} transport into the mitochondria.

6.4 BAP31 Cleavage Regulates Mitochondrial Fission

It is difficult to predict how ER Ca^{2+} signals influence mitochondrial fission because the molecular mechanisms governing mitochondrial fission are themselves poorly understood. It is clear that Drp1 is essential for mitochondrial fission, but the functioning mechanisms of dynamin proteins remains controversial. Dynamins are involved in numerous cellular processes including, endocytosis and vesicle formation, membrane biogenesis, and membrane morphology (Hinshaw, 2000). However, it is unclear whether these GTPases act as molecular switches or as mechanoenzymes that constrict and shape membranes. Genetic studies in yeast identified two novel proteins, Fis1p and Mdv1p, which appear to cooperate with Drp1 to induce fission of mitochondria

(Mozdy et al., 2000; Tieu and Nunnari, 2000). Fis1p is a small integral membrane protein of the OMM that binds and localizes clusters of Drp1 at future sites of membrane fission. In cells lacking Fis1p, mitochondrial fission is blocked and Drp1 aggregates in one or two spots on the surface of mitochondria (Mozdy et al., 2000). Thus, Fis1p is not responsible for recruiting Drp1 to mitochondria but helps organize the dynamin along the organelle. Drp1 recruitment to mitochondria may involve binding to another surface proteins or lipid. Like Drp1, Mdv1p is a soluble protein that is recruited to mitochondria prior to the onset of fission. In cells lacking Mdv1p, Drp1 is still recruited to mitochondria and forms normal clusters along the membrane, but the fission step is blocked (Tieu and Nunnari, 2000). This suggests that Mdv1p acts at a late step in the fission process, perhaps assembling with Drp1 oligomers as a collar around the mitochondrion, which induces membrane curvature and tubule constriction (Shaw and Nunnari, 2002). At this point, Drp1 and Mdv1p may communicate with an inner membrane dynamin, mgm1p, to stimulate cristae reorganization and inner membrane scission. Finally, GTP hydrolysis by Drp1 is thought to finish the fission process by pinching the opposing sides of the OMM together and severing the membrane when contact is made (Shaw and Nunnari, 2002).

Lowering of ER Ca^{2+} stores with TG or b5-BCL-2 blocked p20-induced recruitment of Drp1 to mitochondria and subsequent fission. Thus, it appears that ER Ca^{2+} signals engage the fission process at the level of Drp1 recruitment. Mitochondria that have received this Ca^{2+} signal may expose a Drp1 receptor at the OMM surface. Interestingly, an inner membrane lipid, cardiolipin, has recently been implicated in recruiting tBID to IMM/OMM contact sites (Lutter et al., 2000; Lutter et al., 2001).

Cardiolipin may be flipped to the OMM outer leaflet at these sites, where it binds tBID. It is possible that a similar process recruits Drp1 to the OMM.

Recent work by Hajnoczky and colleagues revealed that agonist-induced (i.e. IP_3 or caffeine) IP_3R and RyR Ca^{2+} spikes rapidly transmit high Ca^{2+} loads to the mitochondria, leading to PTP opening and cyt.c release in cells prestimulated with an apoptotic insult (Szalai et al., 1999). Although it is unlikely that the IP_3R and RyR achieve such an activation state during physiological apoptosis, these experiments demonstrate the ER Ca^{2+} signals can influence MMP. Hajnockzky's group did not report the occurrence of mitochondrial fission following IP_3R/RyR activation, but this could be explained by the fact that many of the experiments were conducted in permeabilized cells. It is noteworthy, however, that a number of discrepancies exist between the nature Ca^{2+} signaling in their experimental system and what is normally observed during apoptosis, and what I observed following p20 expression. For example, a gradual and sustained increase in $[Ca^{2+}]_m$ has been observed during apoptosis (Kruman and Mattson, 1999; Nutt et al., 2002; Nutt et al., 2001; Pinton et al., 2001) and following p20 expressing (Figure 4.4), whereas IP_3R/RyR agonist induce a large and immediate uptake of Ca^{2+} into mitochondria that is rapidly released back to the cytosol (Pacher and Hajnoczky, 2001). In addition, Hajnoczky's group observed large depolarizations in $\Psi\Delta_m$ prior to the release of cyt.c, which are not observed prior to MMP in apoptotic cells (Goldstein et al., 2000) (although small and transient openings in the PTP may be required for MMP). These discrepancies might be explained by the somewhat artificial nature of the ER Ca^{2+} spikes in Hajnoczky's experiments. Nonetheless, matrix accumulation of Ca^{2+} has long been known to stimulate PTP opening (Duchen, 2000), so it could be that p20's ability to induce a sustained increase in $[Ca^{2+}]_m$ induces Drp1 recruitment indirectly via PTP

opening. Unfortunately, I was unable to address the affect of a PTP inhibitor, CsA, on p20-induced fission because this agent was cytotoxic over the course of Adp20 infection (data not shown).

Regardless of how BAP31-regulated Ca^{2+} signals promote Drp1 recruitment to mitochondria, the end result is clear; mitochondria that have undergone fission are highly sensitized to cyt.c release. Fission of the mitochondrial network is one of several morphological changes mitochondria must undergo before MMP (Frank et al., 2001). Caspase-8 cleaved BID (tBID) causes dramatic cristae remodeling (Scorrano et al., 2002), suggesting that in addition to its affects on BAK and BAX, this BH3-only protein also influences the IMM dynamin, OPA1 (the mammalian mgm1p ortholog). A recent report documented that siRNA knockdown of OPA1 induces similar cristae restructuring, followed by mitochondrial fission, cyt.c release and apoptosis (Olichon et al., 2002). Therefore, apoptotic IMM cristae remodeling and Drp1-dependent mitochondrial fission are intimately linked and both processes are critical for cyt.c release (Frank et al., 2001; Scorrano et al., 2002). Dramatic apoptotic cristae remodeling may not be possible within the defined space provided by the OMM and, thus, scission of the OMM may allow mitochondria to reconstruct their interior. Alternatively, BAK/BAX pores might form in the cleavage furrows generated during fission of individual mitochondria. In anycase, these membrane remodeling events seem to be required to mobilize cyt.c stores out of cristae junctions and for BAK and BAX to achieve full activation and oligomerization in OMM. Moreover, Ca^{2+} signals from the ER are paramount to these mitochondrial changes since crBAP31 inhibited active state conformational changes BAX and BAK, and the release cyt.c to the cytosol (Figure 5.4). Thus, caspase-8 cleavage of BID and BAP31 insures that cristae remodeling, organelle fission and BAX/BAK oligomerization

occur simulateously, allowing rapid and efficient release of cyt.c from mitochondria during death receptor induced apoptosis.

6.4 Perspective

Is it really possible that BAP31 can function as an ER cargo receptor, a procaspase activation complex, and a modulator of ER Ca^{2+} release? It is certainly acceptable that BAP31 could play a role in normal cellular processes like protein trafficking and still have a role in apoptosis. cyt.c and endonuclease G already provide examples of such a phenomenon (Cote and Ruiz-Carrillo, 1993; Kadenbach, 1986). In addition, several studies have implicated a role for caspases in cell growth processes (Alam et al., 1999). Evolution might have given genes involved in essential cellular processes a second role in cell death as means to maintain selective pressure against their loss. Similarly, BAP31 is not the first described multifunctional regulator of apoptosis. BCL-2, CED-9, p53, and BAX/BAK are just several examples of proteins that seem to play two or more roles in apoptosis (Huang and Strasser, 2000; Ljungman, 2000; Xue and Horvitz, 1997). In the case of BAP31, localizing the activation of a procaspase to the site of its substrate may be a strategic mechanism to couple the two events in the oncogene pathway.

These are still early days for defining the role of the ER in apoptosis. It is clear that stress in the ER activates a specific, albeit poorly characterized, cell death pathway. However, much remains to be learned about how ER signals, and their regulation by Bcl-2 proteins, contribute to the activation of the death machinery in other apoptosis pathways. This study has provided one example of how events at the ER can regulate

procaspase activation and demonstrated for the first time that caspase-initiated events at the ER (BAP31 cleavage) can directly affect the core mitochondrial pathway. Many questions remain, such as the mechanism of procaspase-8L activation, how BAP31 and p20 regulate ER Ca^{2+} release, and how this Ca^{2+} signal is coupled to mitochondrial fission. It will be important to determine if BAP31's effects on protein trafficking, ER Ca^{2+} release and procaspase-8L processing are somehow related. The successful generation of mice doubly or singly deficient for Bap31 and Bap29 should yield further insight into these issues, and determine the contribution of ER pathways to developmental cell deaths and the p53 tumor suppressor program.

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Original Contributions to Knowledge

1. Identified and cloned the novel procaspase-8 isoform, procaspase-8L.
2. Demonstrated that procaspase-8L is ubiquitously expressed and localized to the cytosolic face of the ER membrane.
3. Demonstrated that procaspase-8L is selectively recruited to the BAP complex following oncogenic signaling by E1A.
4. Documented that procaspase-8L dominant-negative mutants inhibit E1A-induced apoptosis.
5. Using cells deficient in Bap29 and Bap31, I provided evidence that E1A-induced processing of procaspase-8L is dependent of BAP proteins and important for the activation of downstream caspases and apoptosis in this pathway.
6. Together with Mai Nguyen, I demonstrated that expression of a caspase-resistant BAP31 mutant blocks several features of Fas-induced apoptosis, including actin redistribution, membrane blebbing, cell detachment, and cyt.c release.
7. Together with Mai Nguyen and Axel Ducret, I demonstrated that BAP31 specifically interacts with γ-actin and the myosin B heavy chain.
8. Demonstrated that the p20 caspase cleavage fragement of BAP31 induced Ca^{2+} transmission between the ER and mitochondria.
9. Demonstrated that p20-induced Ca^{2+}signals lead to the onset of mitochondrial fission.
10. Demonstrated that p20 sensitizes cells to caspase-8 induced apoptosis by stimulating mitochondrial fission and proposed a model in which two caspase-8

cleavage products, BAP31 and BID, cooperate to induce cyt.c release in the Fas pathway.

11. Demonstrated that p20 and full-length BAP31 can affect apoptosis pathways independently of one another.
12. Provided evidence that BAP31 is an in vivo substrate of caspase-8.