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Apoptosis Induction by the BH3-only Protein BIK Localized at the Endoplasmic Reticulum

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A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ISBN: 0-494-12847-X
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ISBN: 0-494-12847-X

Expectation is a prison
- Robert Fripp

Résumé

Le maintien de l'homéostasie dans un organisme multicellulaire requiert un contrôle strict de la croissance et de la mort par apoptose des cellules. La dérégulation de ces processus est liée à l'apparition de cancers, de maladies neurodégénératives et autoimmunitaires. Dans ce contexte, l'apoptose est critique pour l'élimination des cellules potentiellement dangereuses pour l'organisme. La protéine p53 joue un rôle clé dans cette réponse activant la transcription de plusieurs protéines impliquées dans la réponse apoptotique. Parmi ces protéines se trouve des membres de la famille de BCL-2, protéines intimement liées à la régulation de l'activation des caspases, les protéases apoptotiques.

J'ai découvert que BIK, un homologue pro-apoptotique de BCL-2 contenant seulement un BH3, est induit par p53. La surexpression de BIK induit la voie classique d'activation des caspases, dépendante de la mitochondrie. Cependant, BIK se trouve presque exclusivement dans le réticulum endoplasmique (RE). Différentes expériences *in vitro* et dans des cellules ont indiqué que BIK induit l'apoptose à partir du RE. Puisque les protéines induites par p53 le sont normalement en faible quantité et en groupe, j'ai étudié les événements induits par BIK tôt après son induction. BIK induit des signaux de Ca^{2+} à partir du RE qui causent la fission des mitochondries et le réarrangement de leur cristae. L'induction de ces processus permet à BIK de coopérer avec une autre cible de p53, Noxa, pour rapidement induire l'apoptose.

Ces fonctions de BIK sont liées à son habilité à interagir avec BCL-2, présent en grande quantité à la surface du RE. J'ai donc étudié les interactions entre BIK et BCL-2 à la surface du RE. J'ai constaté que BIK, en présence d'une quantité de BCL-2 insuffisante pour prévenir l'apoptose, prévient l'interaction entre BCL-2 et les protéines qui y sont normalement associées.

En résumé, BIK est un homologue pro-apoptotique de BCL-2 induit par p53 qui fonctionne à partir du RE pour induire l'apoptose régulée par BCL-2. BIK peut coopérer avec d'autre effecteurs apoptotiques en régulant des changements morphologiques de la mitochondrie par des signaux de Ca^{2+}.

Abstract

Tissue homeostasis is maintained through a balance between controlled cell growth and apoptotic cell death. Deregulation of these processes is linked to cancer, auto-immune diseases and neurodegenerative diseases. In that regard, apoptosis is critical to remove cells that are potentially harmful or superfluous. The p53 tumour suppressor is a key regulator of this process. p53 induces apoptosis through the transcriptional activation of multiple proteins among which are members of the BCL-2 family of proteins. This family of proteins lie at the core of this process by controlling molecular events leading to the activation of the executors of the death sentence, namely the caspase family of proteases.

I found that BIK, a member of the pro-apoptotic BH3-only class of BCL-2 homologues, is upregulated by p53. Upon overexpression, BIK induces classical mitochondria-dependent caspase activation. However, BIK is found almost exclusively in the endoplasmic reticulum (ER). Specific targeting of BIK to the ER and *in vitro* experiments indicated that BIK causes cytochrome c release from an ER location. Since BH3-only proteins are normally expressed at low levels and as a cohort, I subsequently investigated early events induced by BIK at the ER. BIK induces ER Ca^{2+} release leading to mitochondrial fission and cristae remodelling long before cytochrome c release. However, BIK and another p53-induced BH3-only protein, Noxa, can potently cooperate to induce rapid cytochrome c release and apoptosis in a mitochondrial fission-dependent manner.

These functions of BIK are closely linked to its ability to interact with BCL-2, present at high levels in the ER of different cell types. I thus studied BIK-BCL-2 interactions at the ER using a cross-linking approach. Interestingly, the induction of BIK in cells expressing BCL-2 at a level that does not prevent apoptosis results in the displacement of several BCL-2 cross-linked products by BIK.

In summary, BIK is a p53-induced BH3-only protein that functions at the ER to induce BCL-2-regulated apoptosis and can cooperate, through ER Ca^{2+} signals, with apoptotic stimuli provided by other BH3-only proteins to induce rapid apoptotic cell death.

Remerciements

First of all, thanks to Gordon for giving me all the freedom and support I needed, being so positive even when things don't work (as they apparently do sometimes), the discussions and everything else.

To Mai, for always being there to help us and being so full of resources

To the members of the Shore lab, Marina, Nhi, Steph, Jaigi, Sonny, you are great friends, I'll miss you

To Rathna, for everything you do for us (including great Indian food...)

À Bachir et Fred Sallman qui m'ont appris comment faire de la recherche et sans qui je ne serais probablement pas ici aujourd'hui. Les heures passées à discuter science avec vous me manquent

Aux ex-BCMCB Nathalie, Céline, Karine, Simon, Charles, Elaine, pour les bon moments passés autour d'une 3P et pour m'aider à garder l'esprit ouvert…

Un merci tout spécial à ma famille, mes parents Jocelyne et Maurice, ma sœur Karine et bien entendu Jennifer, qui m'ont toujours supporté (et même enduré…).

À notre futur bébé…

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Abbreviations

ANT	Adenine Nucleotide Translocase
BH	BCL-2 Homology (domains)
BKA	Bonkgrekic Acid
BMH	*bis*-maleimidohexane
CARD	Caspase Recruitment Domain
CED	Cell Death defective
CsA	Cyclosporin A
DD	Death Domain
DED	Death Effector Domain
DKO	BAX/BAK Double Knockout mice/cells
ER	Endoplasmic reticulum
FADD	Fas Associated protein with a Death Domain
HM	Heavy Membrane fraction
IMM	Inner Mitochondrial Membrane
IP3	Inositol 1,4,5,-triPhosphate
IP3R	IP3 Receptor
LM	Light Membrane fraction
MEF	Mouse Embryonic Fibroblast
OMM	Outer Mitochondrial Membrane
PTP	Permeability Transition Pore
RNAi	RNA interference
RyR	Ryanodine Receptor
SERCA	Sarco-Endoplasmic Ca^{2+} ATPase
siRNA	small inhibitory RNA
TM	Transmembrane domain
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
UPR	Unfolded Protein Response
VDAC	Voltage Dependent Anion Channel

Contribution of Authors

The work presented in this thesis represents the work I carried on the function of the BH3-only protein at the endoplasmic reticulum. I contributed most of the work both experimentally and intellectually with some exceptions as follows:

Chapter 2: M. Germain, J.P. Mathai and G.C. Shore (2002) BH3-only BIK functions at the endoplasmic reticulum to stimulate cytochrome c release from mitochondria. *J. Biol. Chem.* 277: 18053-18060

All the work was directly conducted by myself aside from the generation of the Ad HA-BIK which was done by Jaigi Mathai and Richard Marcellus.

Chapter 3: M. Germain, J.P. Mathai, H. McBride and G.C. Shore (2004) Endoplasmic reticulum BIK initiates DRP1-dependent mitochondrial dynamics and cristae remodelling. Cooperation with Noxa to stimulate cytochrome c release to the cytosol. *submitted*

I did most of the work except the live cell imaging in Figure 3.3 and the electron microscopy in Figure 3.4 that were the work of Heidi McBride at the University of Ottawa. In addition, I created the constructs for the Ad Noxa but the actual generation of the adenoviral vectors was done by Marina Stojanovic.

Chapter 4: This is my own unpublished data except for the experiment in Figure 4.2B and C which was carried by Stephanie Bueler, who was summer student under my supervision in the lab at the time. I am still waiting for the MS results for the identification of the BCL-2-interacting proteins to publish...

Chapter 5: The data on anti-apoptotic BCL-2 proteins interactions was all done by myself, although I should mention the help of Stéphane Acoca who did a bioinformatic screen of BH3 domains which helped me work out the differences between BH3 domains.

Appendix 1: J.P. Mathai, M. Germain and G.C. Shore (2004) Human endoplasmic reticulum BIK is required for p53-induced Ca^{2+}-mediated mitochondrial fission signals and apoptosis. *Submitted*

This work was added as an annex mainly because it provides a validation for my work done on BIK in the context of p53. Most of the work was done by Jaigi Mathai although I contributed intellectually and by providing the framework for BIK action (Chapter 3)

Original Contribution to Knowledge

I would say that my original contribution to knowledge is a better understanding of the function of BCL-2 homologues at the endoplasmic reticulum. By identifying a pathway induced by BIK that leads to sensitization of mitochondria to cytochrome c release through mitochondrial fission and cristae remodelling, this work provides better tools for understanding the role of endoplasmic reticulum during apoptosis. In addition, my work provides insights into the function of BIK through its effects on BCL-2-interacting proteins. Finally, the observed cooperation between BIK and Noxa in the p53 pathway provides a new perspective for understanding the apoptotic activity of p53.

In any case, here is a list for those who need it... My work:

1. Identified BIK as the first BH3-only protein exclusively associated to the endoplasmic reticulum
2. Demonstrated that BIK can activate cytochrome c release and apoptosis from a location at the endoplasmic reticulum rather than at the mitochondria
3. Showed a functional link between mitochondrial fission and cristae remodelling, providing a mechanism for sensitization to cytochrome c release caused by fission
4. Showed that BIK induces Ca^{2+} release from the endoplasmic reticulum which has not been shown before for BH3-only proteins
5. Showed that Ca^{2+} signals elicited by BIK cause mitochondrial fission and cristae remodelling
6. Demonstrated that two p53-induced BH3-only proteins can cooperate to induce apoptosis
7. Demonstrated that BIK and BCL-2 functionally interact at the endoplasmic reticulum
8. Showed that BIK displaces the interaction of BCL-2 with its binding partners at the endoplasmic reticulum

Chapter 1: Introduction

For all forms of life, survival requires a tight balance between proliferation and death. This is true for unicellular organisms but even more crucial for development and homeostasis of multicellular organisms. This balance is achieved through two tightly controlled mechanisms: cell division and programmed cell death. This occurs all through development, shaping organs and other structures (1), and continues through adulthood to remove unwanted cells. One dramatic example is the immune system (2, 3). In order to recognize and destroy invading pathogens, the body has at its service a wide range of specialized cells, including B and T lymphocytes. These circulating lymphocytes are the survivors of a strict screen which filters out unresponsive and self-recognizing cells. A tight control of lymphocyte population must be kept as improper regulation will lead to either impaired immune system or auto-immune diseases. A second example associated to the maintenance of tissue homeostasis is the deletion of cells that are escaping cellular growth control imposed by the organism and are thus potential cancer cells. One of the major regulators of this process is the p53 tumour suppressor (4, 5). In fact, p53 is mutated in roughly 50% of human cancers while other components of this pathway are compromised in the majority of the other cancers. p53 is a transcription factor that can induce either cell cycle arrest or apoptosis, a biochemically defined form of programmed cell death.

In this chapter, I will define the core machinery of the apoptotic process as well as the current understanding of how p53 can trigger this process. Interestingly, this process is likely to be more complex than the paradigm that was prevalent at the time of starting this research postulated. We are now realising the importance of new players that were discovered in the last few years. One such example is the role played by the endoplasmic reticulum (ER) during apoptosis which has started to be unravelled in many laboratories. As a good portion of the work presented here involves some of these new players, they will therefore be introduced below as well.

A general overview of apoptosis

A morphogically defined form of programmed cell death

The term apoptosis was first proposed in 1972 by Kerr and colleagues (6) to describe a form of programmed cell death that was morphologically distinct from necrosis. Hallmarks of apoptosis include nuclear condensation, internucleosomal DNA degradation, cell shrinkage and dismantling into vesicle-like structures that bud from the cell in a process known as blebbing (7, 8). Dying cells are subsequently phagocytosed by macrophages or neighbouring cells (9). In contrast, necrosis is characterized by swelling of the cell which leads to disruption of cellular integrity and release of its potentially harmful content into the extracellular milieu (8). Death by apoptosis thus results in a clean waste disposal which has the added advantage of preventing an inflammatory response that could be deleterious to the organism. Indeed, by turning off the pro-inflammatory state of macrophages, phagocytosed apoptotic cells play a crucial role in the resolution of inflammation (9).

Killer caspases

On the biochemical level, apoptosis is characterized by the activation of a family of cysteine proteases named caspases (7, 10, 11). These are highly specific proteases that have an absolute requirement for an aspartic acid (D) at the P1 position of the cleavage site. Caspase recognition sequence is defined as a four amino-acid xxxD motif (where x is any amino-acid) that varies for different caspases (10). For example, the effector caspases-3 and -7 recognize a DxxD consensus motif. All caspases are present in the cytosol of healthy cells as inactive proenzymes composed of a prodomain, a p20 and a p10 subunit. Activation occurs upon cleavage between the p20 and the p10 subunit (7, 10). Removal of the prodomain is also required for effector caspase activation. As these proteolysis events occur at an aspartate, caspases can be activated in a cascade. Initiator caspases (caspase-2, -8, -9, -10) have a long prodomain that possesses one of two types of interacting domains: the Caspase Recruitment Domain (CARD) in caspase-2 and -9 or the Death Effector Domain (DED) in caspase-8 and -10 (7, 10, 11). These domains allow multiple copies of an initiator

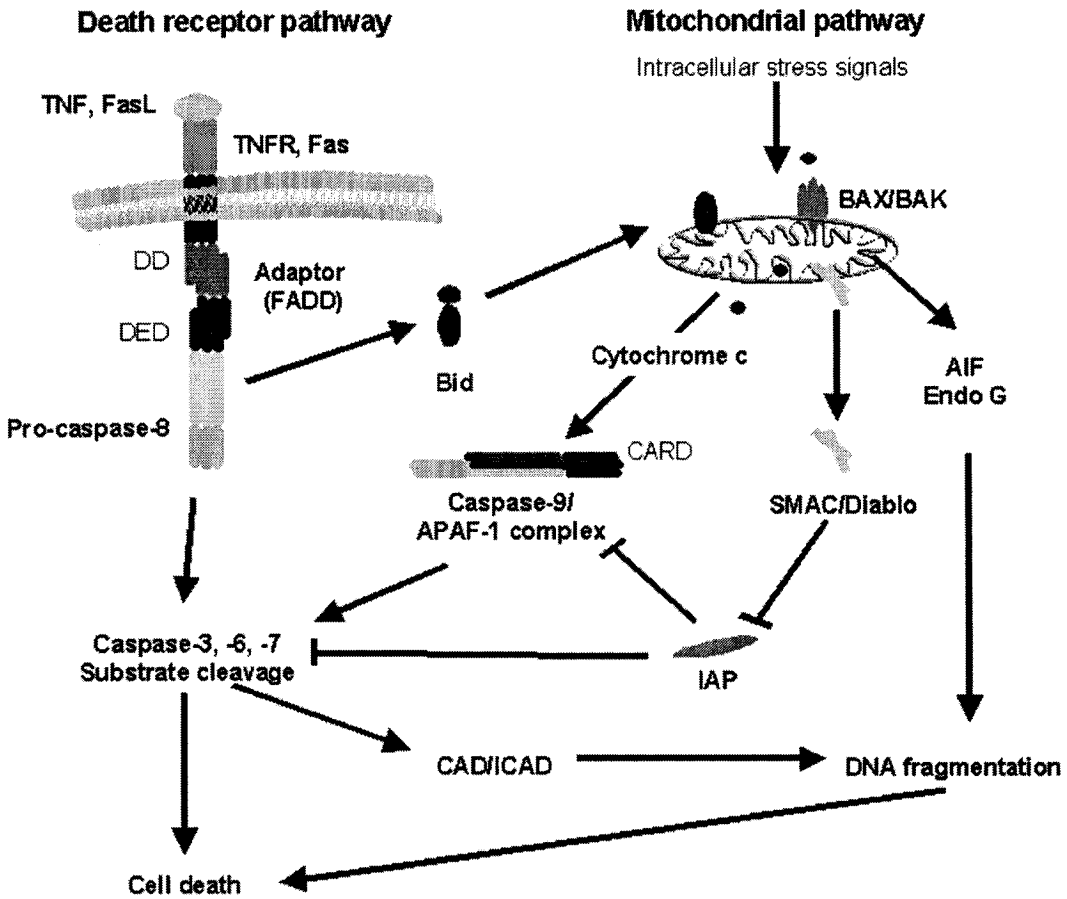

Figure 1.1 ***The major apoptotic pathways.*** Three types of domains mediate the interactions required for the recruitment of initiator caspases at their respective initiation complexes. Upon trimerization of the death receptors by their ligands, protein-protein interactions mediated by Death Domains (DD) and Death Effector Domains (DED) recruit pro-caspase-8 at the death receptors through the adaptor protein FADD. The pro-caspase-9/APAF-1 apoptosome is brought together by the Caspase Recruitment Domain (CARD) present in both molecules. This interaction is triggered by cytochrome c in the presence of ATP. The BCL-2 family regulate the mitochondrial outer membrane permeability. Once cleaved by caspase-8, the BH3-only BID activated pro-apoptotic BAX and BAK which are responsible for the release of cytochrome c as well as the Apoptosis Inducing Factor (AIF) and Endonuclease G (Endo G) from the mitochondrial intermembrane space. AIF and Endo G then migrate to the nucleus where they cause DNA fragmentation. SMAC is another protein released from apoptotic mitochondria. It binds and inactivates the Inhibitors of Apoptosis (IAP) which normally bind to and inactivate caspase-3, -7 and –9.

caspase to be recruited to initiation complexes by binding to adaptor proteins. This induced proximity allows its auto-catalytic cleavage and activation (12). Effector caspases (caspases-3, -6, -7) are activated by the initiator caspases and are responsible for proteolysis of a number of substrates, resulting in the morphological characteristics of apoptosis. Substrates range from DNA repair

enzymes (PARP, DNA-PK_{CS}) to skeletal proteins (Fodrin) and other proteins involved in maintenance of cellular homeostasis (for a detailed review, see (13)). The general feature of caspase cleavage is that it separates the regulatory domain from the catalytic domain, resulting in constitutive activation or inactivation of the protein (7, 10, 11).

How to kill a cell:Major pathways to cell death

Two classical pathways are known to activate the formation of initiator caspase complexes leading to apoptosis (7, 11). These are the death receptor pathway and the mitochondrial/apoptosome pathway (Figure 1.1).

The Death Receptor Pathway

This first pathway is initiated by the binding of extracellular ligands (Fas ligand, TNF, TRAIL) to members of the death receptor family of surface proteins. This triggers trimerization and activation of the receptor which recruits adaptor proteins via their Death Domain (DD). These adaptors, such as FADD, possess both a DD and a DED that can bind to the Fas DD and procaspase-8 DED respectively. Binding of procaspase-8 to this complex allows its activation. This is thought to occur through the autocatalytic activation of the enzyme induced by the close proximity of multiple copies of the protein (14). However, recent evidence suggest that removal of the prodomain might not be required for caspase activity (reviewed in (14)).

The Apoptosome

The second, intrinsic, pathway relies on the activation of caspase-9 through the formation of a complex called the apoptosome. This complex was first purified as an activity capable of activating caspase-3 *in vitro*. The apoptosome is a 1 MDa protein complex containing multiple copies of procaspase-9, the adaptor protein APAF-1, cytochrome c and requires ATP to be activated (15). Cytochrome c being part of the respiratory chain in the mitochondria required for ATP production, the discovery of this unsuspected killer was quite a shock in the field (16). Further work confirmed that during apoptosis, cytochrome c leaks out of the mitochondrial intermembrane space, along with other pro-apoptotic proteins, to

induce caspase activation through the apoptosome (reviewed in (7, 11)). This discovery rapidly became one of the central models of caspase induction, although some believe that it acts as an amplifier rather that an initiator (17, 18). With the idea that mitochondria could act as "the central executioner" during apoptosis (19), a great amount of research became focussed on the mechanism of cytochrome c release.

Opening the "central executioner"

The permeability transition pore

One of the hypotheses as to how cytochrome c is released from mitochondria during apoptosis is through the induction of the Permeability Transition Pore (PTP). The PTP is a pore in the mitochondrial inner membrane that can let through solutes up to 1500 Da, leading to depolarization and swelling of mitochondria and possibly causing rupture of the outer membrane and release of cytochrome c (20-22). Its core is formed by two proteins: the Adenine Nucleotide Translocase (ANT) and cyclophylin D (21). ANT, located in the inner mitochondrial membrane (IMM), regulates the ATP and ADP flux through the IMM while cyclophylin D is a peptidyl prolyl *cis/trans* isomerase located in the matrix (23, 24). The Voltage Dependent Anion Channel (VDAC), an outer mitochondrial membrane (OMM) protein which forms pores allowing transport of solutes across the OMM (25) could also be part of the PTP (22). The molecular composition of the pore is however still debated. In fact, the recent report of an ANT knockout animal suggested that ANT might not be absolutely required for PTP formation (26). Despite the elusive molecular nature of the pore, its physical properties are well known (21). Of note, the PTP is induced by mitochondrial Ca^{2+} overload which can lead to cell death (22).

Different experiments have suggested a role for the PTP in apoptosis. First, inhibitors of components of the PTP such as the cyclophylin D inhibitor Cyclosporine A (CsA) or the ANT inhibitor Bonkgrekic acid (BKA) have been shown to prevent apoptosis in various settings (27). Second, a depolarization of mitochondria indicative of the PTP opening has been reported in cells undergoing

apoptosis (27). In addition, members of the BCL-2 family (see below) have been shown to interact with components of the pore (28-31). However, PTP inhibitors are not very selective and it is still debated whether depolarization occurs upstream of cytochrome c release (32). Furthermore, in many cases PTP opening has been associated to necrosis rather than apoptosis (33). Taking this into consideration, along with evidence for the involvement of another pathway described below, PTP opening is unlikely to be the major pathway for cytochrome c release. This does not however exclude a role for PTP components in this process. Indeed, accumulating evidence suggest a role for PTP components in modulating mitochondrial response to pro-apoptotic stimuli. One example of this is the modulation of apoptosis by VDAC through the control of mitochondrial Ca^{2+} signalling ((34), see below).

BCL-2 regulated pores

A number of findings have suggested an alternative explanation for mitochondrial permeabilisation during apoptosis, pointing to a role for the BCL-2 family of proteins in this process. First, it was known that BCL-2 and BCL-X_L were localized at the mitochondria and the ER/nuclear membrane where they could antagonize pro-apoptotic members of the same family (35, 36). Second, the resolution of the structure of BCL-X_L revealed a striking homology with pore forming bacterial toxins (37) and it was later shown that BCL-2 homologues could indeed form pores in synthetic lipid bilayers (30, 38). Another indication came with the discovery that, yet another member of the family, Bid, was cleaved by caspase-8 triggering its translocation to mitochondria and promoting cytochrome c release (39). The hypothesis, which is now prevalent, is thus that BCL-2 family members regulate cytochrome c release from mitochondria. I shall thus describe further these important regulators of the apoptotic process.

The BCL-2 family of apoptotic regulators

BCL-2 homologues can be divided in three groups according to their effect on apoptosis induction: anti-apoptotic, pro-apoptotic and the BH3-only proteins (reviewed in 7, 40, 41). BCL-2 proteins can modulate each other's function

through dimerization/oligomerization and this indeed plays a crucial role for their activity (7, 40). Different models have been proposed to explain the functional relationship between the different classes of BCL-2 homologues (Figure 1.2B). In all these models, protein-protein interactions between the three groups of BCL-2 proteins play a prominent role.

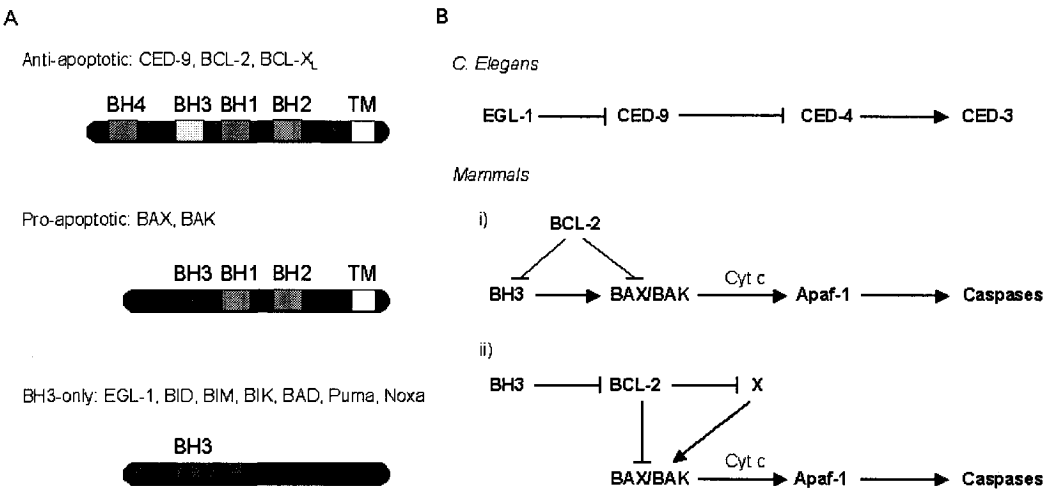

Figure 1.2 ***The BCL-2 family of apoptotic regulators.*** (A) BCL-2 homologues are identified by the presence of at least one of the BCL-2 homology (BH) domains. The three classes of BCL-2 proteins differ in their conserved BH domain as well as by their pro- or anti-apoptotic properties. (B) Models for the activity of BCL-2 homologues. In mammalian cells, tBID binds preferentially BAX/BAK to induce their activation (*i*). Model (*ii*) has been suggested because other BH3-only bind preferentially anti-apoptotic BCL-2 homologues (BCL-2). In that case, BCL-2 could sequester a BAX-activating factor (X) that is liberated upon induction of BH3-only proteins.

Anti-apoptotic, pro-apoptotic and the BCL-2 Homology Domains

The anti-apoptotic proteins are BCL-2 itself, as well as BCL-W, BCL-X_L, MCL-1 and BFL-1. With the exception of BFL-1 (A1) and MCL-1, they all contain four conserved BCL-2 Homology (BH) domains which correspond to 20-25 amino-acid α-helices (Figure 1.2A; 1.3). The BH4 domain has been shown to be required for their anti-apoptotic activity although its exact function remains unclear (42). It should be noted that although the anti-apoptotic activity is always associated with a N-terminal domain, the BH4 is not the only N-terminal anti-apoptotic domain as BFL-1 and MCL-1 contain different N-terminal domains but are still anti-apoptotic (Figure 1.3). The BH3, BH1 and BH2 helices from multi-domain BCL-2 homologues form an hydrophobic pocket in which the BH3 of BH3-only proteins can bind (43). The BH1-3 form therefore two functional modules

constituting the structural basis for the interactions among BCL-2 homologues (40, 44). The BH3 is required for the killing activity of pro-apoptotic family members, which can be classified in two distinct subgroups: pro-apoptotic and BH3-only. The pro-apoptotic BAX and BAK possess the BH1-3 pocket but they lack the anti-apoptotic BH4 (as both proteins are very similar in structure and function, they will be referred to as BAX/BAK). These two proteins are required for cytochrome c release and apoptosis. Mice deficient in both proteins (double knock-out (DKO) mice) are resistant to a vast number of apoptotic stimuli, lending strong support for the critical role of BCL-2 family proteins in controlling mitochondrial integrity (45-47). The exact mechanism of action of BAX and BAK however, is still elusive. Both proteins can form oligomers that have been reported to be constituted of at least four BAX/BAK molecules (48-50). These may either constitute the pore themselves or act on some other mitochondrial pore forming proteins such as VDAC. BAX has also been suggested to form lipidic pores that are influenced by membrane curvature (51, 52) which tBID can directly alter (53).

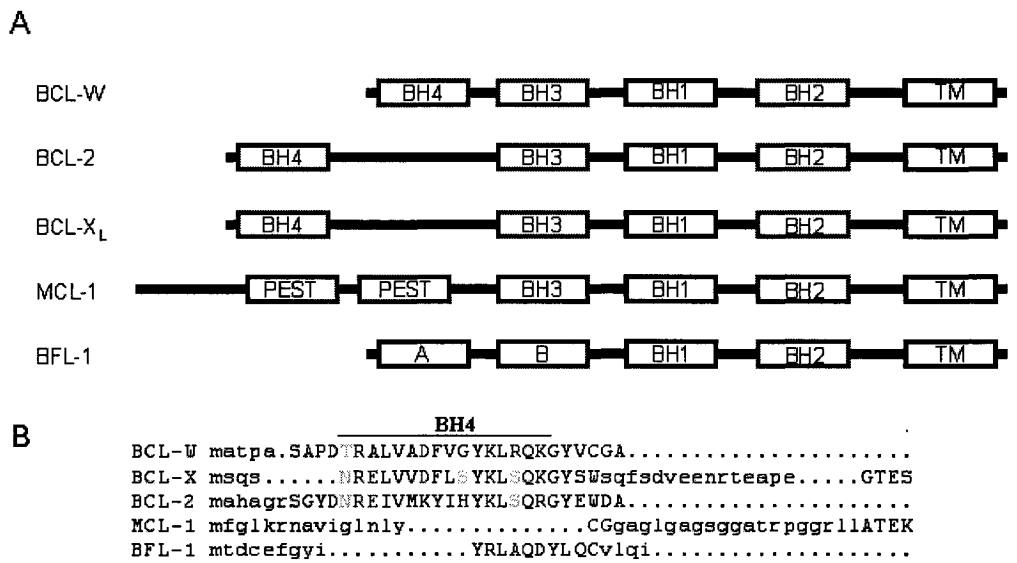

Figure 1.3 ***Anti-apoptotic BCL-2 proteins.*** (A) Domain structure (B) Sequence alignment of the the N-terminus showing BH4 conservation. Alignment was performed using Dialign2

BAK is constitutively localized at the surface of the mitochondria while BAX normally resides inactive in the cytosol. Both proteins are present as a monomer

until, upon stimulation, they undergo a conformational change and oligomerize at the surface of the mitochondria. This step is regulated in both cases by the N-terminus of the protein which becomes exposed and thus detectable by epitope-specific antibodies (49, 54, 55).

The BH3-Only Proteins

This is where BID, the BCL-2 homologue activated by caspase-8, comes into play. BID has been instrumental in developing our current vision of the mechanism of action of BCL-2 proteins. BID is part of the third class of BCL-2 homologues, the BH3-only proteins, which possess only the BH3 killing domain. Some recently discovered BCL-2 homologues, such as Spike (56) or BCL-Rambo (57), carry other functional domains regulating their activity. In contrast, classical BH3-Only proteins such as BID or BIK contain only the BH3 as a functional domain and can be regulated through several mechanism (reviewed in 36, 40, 58). One way of regulating the activity of BH3-only proteins is through transcriptional activation. In normal cells, many BH3-only proteins are undetectable at the protein level. The upregulation of these proteins upon stress result in the production of a constitutively active BH3-only protein that can dominantly induce apoptosis. Examples of these include Puma, Noxa, BIK and HRK (40, 58, 59). Other BH3-only proteins such as BID, BIM and BAD are constitutively expressed and thus need to be kept inactive in living cells. In the case of BAD, phosphorylation at several residues results in the cytosolic sequestration of the protein by 14-3-3 proteins (60). Similarly, BIM is sequestered on the microtubules through its interaction with the LC8 dynein light chain (61). Upon induction of apoptosis, both proteins are released and translocate to mitochondria. BID normally resides inactive in the cytosol where it is activated upon cleavage by caspase-8, to generate truncated BID (tBID) (39), allowing tBID to bind other BCL-2 family members.

Models for BH3-only function

The proteolysis of BID by caspase-8 also triggers tBID translocation to the mitochondria where it impinges on mitochondrial function. At the mitochondria, tBID binds to BAX/BAK, inducing their oligomerization and subsequent

cytochrome c release (50). This can be blocked by anti-apoptotic BCL-2 in two ways (likely depending on the ratio of BAX/BAK and BCL-2). Overexpressed BCL-2 is able to bind to the active conformer of BAK and block its subsequent oligomerization and cytochrome c release (49). BCL-2 can also bind to tBID and sequester it away from BAX/BAK, preventing their activation (62). In addition, tBID could induce a remodelling of the mitochondrial cristae, allowing for more efficient cytochrome c release (63). However, with the possible exception of BIM, BID is the only known BH3-only with a strong affinity for BAX/BAK, the others interacting preferentially with anti-apoptotic BCL-2 homologues (40, 62). In addition, of all tested BH3-only proteins and peptides, tBID (and its BH3 peptide), display the most robust *in vitro* cytochrome c release activity (S. Ruffolo and GC Shore, unpublished results, (62)). These results indicate that tBID might not behave in the same way as other BH3-only proteins. Indeed, other models for the action of BCL-2 homologues have been proposed based on the work in the nematode *Caenorhabditis elegans*.

One of the first tools for the analysis of programmed cell death has been the genetic analysis of *C. elegans* initiated by Dr. Sidney Brenner, John Sulston and H. Robert Horvitz who received a Nobel prize for their work in 2002 (reviewed in (58, 64)). In the hermaphrodite form, the fate of each of its 1090 cells is known and easy to follow using simple microscopy techniques. It is thus known that 131 of its cells undergo apoptosis through the course of development (64). Mutants with defects in this program (Cell Death defective (CED) mutants) have been identified and the genetic pathway mapped (Figure 1.2B). The pathway comprises Egl-1, Ced-9, Ced-4 and Ced-3, the other Ced genes being required for proper engulfment by neighbouring cells (64). The major breakthrough came with the realization that CED-9 protein is an anti-apoptotic BCL-2 homologue while CED-3 is the caspase responsible for the execution of the cell. In *C. elegans*, CED-9 normally sequesters and inactivates CED-4, the adaptor for CED-3. Upon induction of apoptosis, the CED-9/CED-4 interaction is disrupted and CED-3 can be activated (58, 64). This was later found to be achieved by the BH3-only EGL-1 which is transcriptionaly induced in dying cells (65). However, some

discrepancies exist between *C. elegans* and mammals. First, the long awaited CED-4 homologue APAF-1 does not bind BCL-2 (7, 58). Secondly, cytochrome c does not seem to be required in *C. elegans*. In fact, CED-4 lacks the WD40 repeats found in APAF-1 that are required for cytochrome c binding (7, 58). In addition, BAX and BAK cause somewhat of a problem as they are required for cytochrome c release and apoptosis in mammalian cells but they have no known orthologue in *C. elegans*. Several models have therefore been proposed to explain apoptosis induction in mammals. The first one best describes the behaviour of tBID, as it can directly bind to BAX/BAK to activate them (Figure 1.2B(i)). Other hypotheses are based on the *C. elegans* paradigm. In the model shown in Figure 1.2B(ii), BH3-only proteins activate apoptosis by relieving the inhibition of a "BAX activating factor" sequestered by BCL-2. Activation of a CED-4-like pathway leading to caspase activation upstream of the mitochondria has also been proposed. The fact that most BH3-only proteins bind strongly to anti-apoptotic BCL-2 homologues has lent support to this model. Another explanation for the variation in affinity of the BH3 domains for BAX and BCL-2 came from *in vitro* work from Korsmeyer's group (62). They proposed that there are two types of BH3 domains: the activators (binding BAX/BAK) and the sensitizers (binding anti-apoptotic BCL-2 proteins and freeing activators). BIM and tBID would be examples of the former while BAD and BIK examples of the latter.

The debate is still open concerning the exact mechanism of action of BCL-2 homologues. There is however a consensus on some aspects. BAX/BAK are required for cytochrome c release and induction of apoptosis. BH3-only and anti-apoptotic BCL-2 homologues act upstream of them and regulate each other through direct interactions mediated by the BH3 domain of BH3-only proteins. There are likely at least two different types of BH3 domains that have different mechanisms of action. In addition, BCL-2 homologues are influenced and influence several other intertwined events that lead to apoptosis, one of which is ER Ca^{2+} signalling.

The role of calcium in apoptosis

General considerations on calcium signalling

Ca^{2+} signalling is involved in a wide variety of cellular functions ranging from cell growth and differentiation to muscle contraction (reviewed in (66, 67)). The main Ca^{2+} store in cells is the ER. Various channels mediate ER Ca^{2+} release, among which the inositol(1,4,5)triphosphate receptors (IP3R) and ryanodyne receptors (RyR) are the best studied (Figure 1.4). IP3R are activated by IP3 while the muscle RyR receptors are activated through a voltage gated channel in the plasma membrane. In addition, both channels are opened by Ca^{2+} in a process termed Ca^{2+}-induced Ca^{2+} release. This process is fundamental for the generation of cytosolic Ca^{2+} signals. However, above a certain concentration, Ca^{2+} causes the closure of the channel, terminating the signal. Cytosolic Ca^{2+} is then pumped back to the ER by sarco-endoplasmic reticulum ATPase (SERCA) pump. The plasma membrane Ca^{2+}-ATPase (PMCA) pump and Ca^{2+}/Na exchangers will also pump cytosolic Ca^{2+} out of the cell. In addition, mitochondria play a central role in the regulation of Ca^{2+} signals (see below).

Calcium signalling during cell death

It has been known for a long time that Ca^{+2} overload, especially in the mitochondria where it can induce opening of the PTP, induces cell death (22, 68). In pathological situations such as during ischemia/reperfusion, Ca^{2+} overload causes activation of catabolic enzymes, production of free radicals and substantial alterations in organelle structure and function (22, 68). This results in a generalized breakdown of cellular functions and necrotic cell death. A number of observations also suggested a role for Ca^{2+} signalling during apoptosis. The first hint came with the realisation that Ca^{2+}-dependent nucleases were involved in the internucleosomal degradation of nuclear DNA, observed as a DNA ladder on an agarose gel. In addition, various pro-apoptotic stimuli such as ceramides, glucocorticoids and UV radiation have been reported to involve Ca^{2+} signalling upstream of the mitochondria. It has also been shown that lymphocytes deficient

in IP3 receptors, one of the two major classes of ER Ca^{2+} release channel, were resistant to apoptosis induction by a variety of agents (69, 70).

BCL-2 Homologues and calcium

Indeed, a direct link seems to exist between Ca^{2+} homeostasis and BCL-2 family members. As described above, most of the work on these proteins has been done on their mitochondrial functions. It has been known for a long time, however, that at least some BCL-2 homologues can be found in other organelles such as the ER. The ER playing an important role as the major Ca^{2+} store in the cell, the link between ER Ca^{2+} and BCL-2 has extensively been studied. Some studies reported no effect or an increase in ER Ca^{2+} stores upon BCL-2 overexpression, while other studies indicated that BCL-2 can decrease Ca^{2+} stores. Recent reports further support a role for BCL-2 in decreasing ER Ca^{2+} stores, rendering cells more resistant to death stimuli involving Ca^{2+} (68, 71, 72). Interestingly, while deletion of BCL-2 increases ER Ca^{2+} stores (73), pro-apoptotic BAX and BAK seem to have the opposite effect, as DKO MEFs have lower ER Ca^{2+} stores (74). Replenishing ER Ca^{2+} by overexpressing the SERCA pump led to a sensitization of these otherwise resistant cells to pro-apoptotic agents known to necessitate Ca^{2+} for their action. However, the only way to reinstate tBID killing in these cells was to reintroduce BAX at the mitochondria (74). These results suggested the presence of two types of signals leading to mitochondrial dysfunction: ER Ca^{2+}-dependent signals (ceramides and H_2O_2) and BH3-only-dependent signals (tBID). It should however be noted that, as stated above, tBID might not be representative of all BH3-only proteins. Overall, the study of BCL-2 homologues in the context of Ca^{2+} signalling indicated that both pro- and anti-apoptotic BCL-2 homologues influence ER Ca^{2+} stores under normal survival conditions.

On the other hand, once the pro-apoptotic BAX and BAK have been activated, they can target the ER and cause massive release of its Ca^{2+} content (75-77), suggesting a possible mechanism for the Ca^{2+} flux observed in apoptotic cells. Another explanation for the sustained Ca^{2+} efflux from the ER during apoptosis could be related to the discovery that cytochrome c binds to IP3R at the ER, blocking its closure triggered by high cytosolic Ca^{2+} concentration (78). The

authors of this study suggested that the initial release of a small amount of cytochrome c from the mitochondria could induce irreversible opening of the IP3 receptor channels. This would generate sustained oscillatory cytosolic Ca^{2+} acting as a positive feedback loop to cause more cytochrome c release.

Downstream of calcium signals

In addition to the already mentioned endonucleases and the PTP, several effectors could potentially contribute to the activation of the apoptotic cascade downstream of ER Ca^{2+} release (reviewed in (8, 68)). For example, the Ca^{2+}/calmodulin dependent protein phosphatase calcineurin has been shown to dephosphorylate the BH3-only BAD, leading to its translocation to the mitochondria and cytochrome c release. Another class of Ca^{2+}-dependent enzymes that could play a role in apoptosis are the calpain proteases. Calpains share some of their substrates with caspases and have been shown to activate caspase-12 following ER stress (79, 80). However, calpains have been implicated in cell survival as well.

More important than these Ca^{2+}-activated signalling pathways might be the interplay between mitochondria and ER, which has been shown to play a crucial role during apoptosis. Extensive work on this subject has been carried by the groups of György Hajnoczky and Rosario Rizzuto (34, 71, 81-83). In intact cells, a portion of the mitochondrial network lies in close proximity to the ER and plays a crucial role in the IP3 driven calcium signals by taking up the released Ca^{2+} (84). This normally leads to an increase in energy production which is required to sustain growth and differentiation (21, 68). In a pro-apoptotic setting, this can however result in a transient PTP opening and cytochrome c release. As pro-apoptotic signals are likely to come from one region of the cell, they need to be relayed to the rest of the cell in order to induce rapid and complete caspase activation. Because of the nature of the apoptotic Ca^{2+} signals, propagating themselves in waves through the mitochondrial network, they are a likely candidate for spreading this signal through the cell by synchronizing cytochrome c release. The fact that two apoptosis-related proteins that can increase the permeability of the mitochondrial outer membrane, namely VDAC and tBID, can

potentiate these events (34, 81) further suggest a role for ER-mitochondria Ca^{2+} cross-talk during apoptosis.

Mitochondrial fission and fusion

Fission and apoptosis

In parallel, it was shown that ceramide causes release of ER Ca^{2+} which is taken up by the mitochondria, affecting their morphology (71). These events were shown to be required for ceramide-induced apoptosis as any condition that reduced mitochondrial Ca^{2+} uptake blocked the pro-apoptotic effects of ceramides. In fact, a role for Ca^{2+} in altering mitochondrial morphology has been known since 1980 (85), but it is not until the realization in 2001 that mitochondrial fission is involved in apoptosis induction (86) that things started to fall into place. Direct evidence for a link between Ca^{2+} signalling and mitochondrial fission came with the report that p20, the pro-apoptotic fragment of the ER protein BAP31, causes Ca^{2+}-induced mitochondrial fission. This sensitizes cells to further pro-apoptotic stimuli such as tBID (87).

So what is mitochondrial fission? Classical textbook images describe mitochondria as small cigar shaped organelles constituted of two membranes separated by a region called the inter-membrane space. The inner membrane is shown with many invaginations forming the cristae that is freely accessible to the inter-membrane space. More powerful imaging techniques revealed that this is likely to be somewhat of a misrepresentation for two reasons. First, the cristae is likely to constitute a compartment distinct from the inter-membrane space due to a very narrow space separating these two compartments (88). Second, immunofluorescences techniques and time-lapse microscopy have depicted mitochondria as organized in a highly interconnected and dynamic network shaped by numerous fission and fusion events (89, 90). This has direct consequences on mitochondrial function both in healthy cells and apoptosis. For example, Ca^{2+} diffusion within the mitochondrial matrix is thought to be more efficient in tubules, which affect mitochondrial response to Ca^{2+}. Another consequence of mitochondrial structure is on cytochrome c release. In intact cells,

most cytochrome c is in the cristae, preventing its release to the cytosol even if the OMM is permeabilized (74, 91). Remodelling of the cristae is thus required in order to allow diffusion of cytochrome c to the inter-membrane space where it can be released upon permeabilization of the OMM. tBID has been shown to cause such a cristae remodelling (63). As coordinated fission of both mitochondrial membranes is required for proper organelle function and that fission is required for cytochrome release, components of the fission machinery are also attractive candidates for cristae remodelling during apoptosis (92).

Physiologically, fission and fusion are important for DNA exchanges between mitochondria and repartition of mitochondria among daughter cells (89, 90). In apoptotic cells, the mitochondrial network is broken up in small round organelles, in a process termed mitochondrial fission (86).

The fission and fusion machineries

Components of the fission and fusion machineries have first been studied in yeast but many mammalian homologues have been identified in the last few years. Yeast DNM1, as well as its counterpart in other organisms such as human DRP1, are GTPases of the dynamin superfamily that play a key role in the fission process (89, 90, 93). Genetic deletion of DNM1 in yeast results in extremely interconnected and fused mitochondria, indicative of a lack of fission. A similar phenotype was observed in human cells using dominant negative DRP1 in which the GTPase activity was abolished by mutation of lysine 38. On the mitochondrial surface, these proteins are part of a larger complex involving at least two other proteins, yeast FIS1 and MDV1 (mammalian homologues are known only for FIS1) (89, 90, 93). FIS1 is an integral OMM protein that recruits DNM1 at mitochondria fission sites. MDV1 is likely to be an adaptor that bridges FIS1-DNM1 interactions. In addition to its interactions with the fission machinery, DNM1 can form homo-oligomers. As is the case with classical dynamins, this oligomerization might be important for the actual fission event.

The first fusion gene discovered was a *Drosophila melanogaster* protein termed fuzzy onion (fzo). FZO1 is a large transmembrane GTPase distinct from dynamins (89, 90, 93). The two mammalian homologues of FZO1 are mitofusin

(MFN)1 and 2. Knock-out of these proteins causes massive fragmentation of the mitochondrial network, indicative of an important role for these proteins in the fusion process (89, 90). As with fission proteins, MFN1 and MFN2 can form multiple homotypic and heterotypic interactions which differentially regulate their function. A second yeast fusion protein is UGO1 which spans the OMM with the N-terminus in the cytosol, but its role is still elusive (94). A third possible component of the fusion machinery is yeast MGM1 and its mammalian counterpart OPA1. OPA1 is localized in the intermembrane space, tightly associated to the IMM (89, 90, 95). However, OPA1 could play other roles such as regulating the IMM structure (90). Its downregulation using siRNA has been reported to cause drastic changes in mitochondrial morphology (95) and cristae structure (95, 96), as well as cytochrome c release and apoptosis (96). OPA1 could also be required to synchronize the fusion of both membranes. Other candidates for regulating IMM fusion are the MFNs which span the OMM twice with both ends facing the cytosol. Its short inter-membrane segment has been shown to be important for co-ordinated fusion of both membranes (89, 90). On the other hand, nothing is known on how division of the two membranes are co-ordinated during the fission process. This is however a very important question as fission and fusion of both membranes must be coordinated for proper mitochondrial function.

Other proteins could also modulate the activity of the fission and fusion machinery. One example, at least in cells undergoing apoptosis, is BAX/BAK. These two proteins have been found in clusters of several thousands of molecules on the surface of the mitochondria (97). Further work revealed that these foci corresponded with sites of DRP1 recruitment (98). In addition BAX was shown to colocalize with MFN2 (98). BAX can however be found at these sites even if DRP1 recruitment is inhibited using a dominant negative DRP1. Strikingly though, BAX could not induce cytochome c release in these conditions (86, 98). Together with experiments looking at Ca^{2+} and fission, these results indicate a functional link between mitochondrial fission and apoptosis.

These are the major players involved in the induction of apoptosis but how are they activated in a physiological setting? Various pathways such as induction of the death receptors or ER stress response lead to activation of the apoptotic machinery. Deregulated induction of oncogenes will also induce an apoptotic response to prevent the generation of cancerous cells. One important mediator of oncogene-induced apoptosis is the p53 pathway.

The p53 network

The p53 tumour suppressor lies at the crossroads of multiple stress signals such as DNA damage, oncogene activation, hypoxia and ribonucleotide depletion (4, 5). Activation of p53 can lead to multiple outcomes: cell cycle arrest, DNA repair, senescence or apoptosis. It is still unclear however how the actual fate of the cell is decided, although integration of multiple death and survival signals is likely to play a key role. An indication of this is that tumour cells are more sensitive to p53-induced apoptosis than their normal counterpart. Anyhow, the pro-apoptotic function of p53 is thought to play a major role in its anti-tumour activity (4).

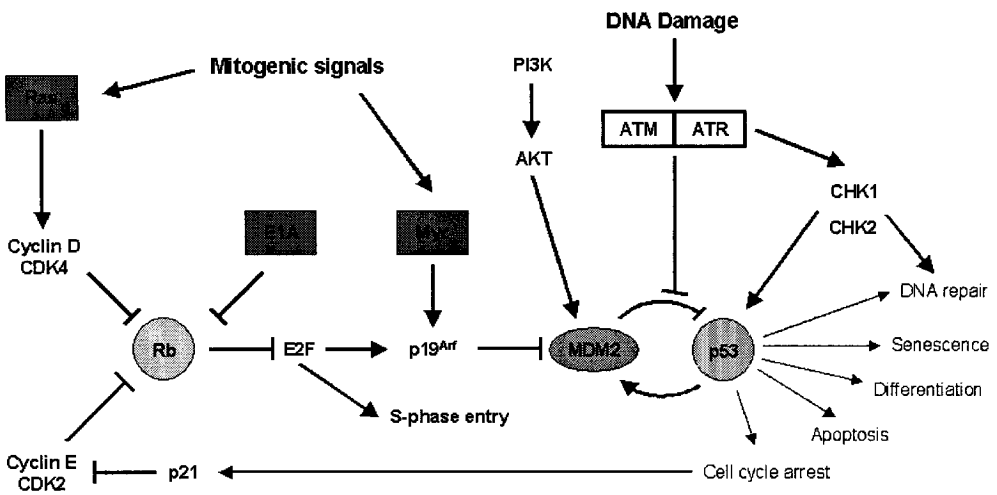

Figure 1.5 ***The p53 Network.*** Multiple signals feed into the core MDM2/p53 regulatory element through direct interactions and post-translationnal modifications, leading to p53 activation. While DNA damage activates the ATM and ATR kinases responsible for the phosphorylation of both MDM2 and p53, mitogenic signals such as activated oncogenes induce the activation of $p19^{ARF}$. $p19^{ARF}$ directly binds to MDM2, inhibiting its function.

Activation of p53

p53 is a sequence-specific tetrameric transcription factor. It is normally found at low levels in cells due to its binding to MDM2, an E3 ubiquitin ligase, which triggers its export from the nucleus and degradation by the proteosome. Stabilisation and activation of its transcriptional activity can be achieved through different mechanisms depending on the stress signal (reviwed in (4, 99-101)). All these signals rely on extensive post-translational modification of p53 such as phosphorylation, acetylation or poly(ADP-ribosyl)ation. For example, DNA damage induced by ionizing radiation activates the PI3K-related kinase ATM (102, 103). ATM phosphorylates both p53 (on serine 15) and MDM2 (on serine 395) resulting in the disruption of the interaction of MDM2 and p53 and the stabilization of the latter in the nucleus (Figure 1.5). Another related kinase, ATR, plays a similar role in signalling UV-induced lesions. In addition, ATM and ATR activate CHK1 and CHK2, two kinases involved in G2 arrest but that also phosphorylate and activate p53 (103).

A second well defined pathway for p53 activation is the one triggered by activated oncogenes (Figure 1.5, Reviewed in (101, 102)). Oncogenes such as E1A or Myc induce the activation of $p19^{ARF}$ which binds to, and antagonizes the function of MDM2. E1A is an adenoviral protein that triggers cell cycle entry for the purpose of viral replication. It functions by inhibiting Rb which normally sequesters E2F transcription factors and prevents their activation. E2Fs induce the transcription of genes required to promote the S phase of the cell cycle. When expressed at high levels, E2Fs are also able to activate $p19^{ARF}$. Abnormal entry in the cell cycle induced by activated oncogenes is therefore linked to the induction of $p19^{ARF}$ and p53-dependent apoptosis. However, normal E2F activation and cell cycle entry is not sufficient for sustained $p19^{ARF}$ activation and apoptosis, additional signals seem to be required. This $p19^{ARF}$/MDM2/p53 module is paramount in preventing tumour formation as it is inactivated in a wide variety of cancers.

Downstream effectors

p53-regulated activities	Target genes
Autoregulation	MDM2
Cell cycle and DNA repair	GADD45, p21
Apoptosis	
BCL-2 homologues	BAX, BAK Noxa, Puma, BIK p53AIP1, p53DINP1
Other apoptotic inducers	PERP, PIDD, APAF1 Fas, Fas-L

Table 1.1 **Regulation of death and survival pathways by p53 through transcriptional regulation of various genes.** The table shows a partial selection of p53-induced genes (Adapted from (4)2}4) and references therein).

Once activated, p53 induces the transcription of a wide variety of genes involved in processes such as cell cycle arrest, DNA repair, apoptosis as well as autoregulatory genes (MDM2, which decreases p53 activation; see table 1.1)(4). Microarray analysis of p53-induced genes suggested that while some, such as the p21 cell cycle inhibitor, seem to be universally induced others may be more cell or stress specific (104, 105). Variation in the set of genes induced by p53 according to the cellular context could hold the key to understanding the various possible outcomes of p53 induction. For example, induction of p53AIP1 has been reported to occur in cells undergoing p53-dependent apoptosis but not during cell cycle arrest (106). This differential activation was associated with phosphorylation of serine 46 of p53 by a protein complex containing p53DINP1 (106, 107). As stated previously, signals feed on the p53 network at different levels, allowing the integration of multiple survival and apoptotic signals. It could therefore be hypothesised that the serine 56 phosphorylation represents such a pathway which activation would be required for p53 to induce the set of proteins responsible for apoptosis induction. However, a number of proteins with potent killing activities are activated irrespective of the cell fate, leaving the debate open. The presence of activated pro-apoptotic genes in surviving cells also suggests these apoptotic inducers might not be as potent killers when expressed at

endogenous levels than what is suggested by overexpression studies. p53-induced genes likely work as a cohort rather than individually to induce apoptosis.

In addition to its role as a transcriptional activator, p53 can repress genes such as anti-apoptotic BCL-2 (4). It has also been shown to be able to induce apoptosis independent of transcription. More specifically, p53 has been shown to induce caspase-8 independently of the death receptors (108) and to localize to mitochondria where it can perform some pro-apoptotic functions such as BAX activation (109, 110). The relevance of this transcription-independent pathway remains however unclear.

Regulation of BCL-2 proteins by p53

A number of BCL-2 family proteins have been shown to be regulated by p53. Among these is the already mentioned downregulation of BCL-2. Pro-apoptotic BAX and BAK have both been shown to be upregulated by p53 (4, 111). As stated previously, BH3-only proteins seem to act at the core of the execution machinery as they are required for BAX/BAK activation. It is therefore not surprising that two BH3-only proteins, namely Noxa and Puma, have been shown to be upregulated by p53 (112-114). Subsequent knock-out experiments showed that both proteins were required for p53-induced apoptosis, at least in some models (115-119). Both Noxa and Puma are localized to mitochondria where they can induce cytochrome c release and apoptosis upon overexpression (112-114). Together with the current knowledge on the BCL-2 family and the fact that BAX/BAK double knockout cells are resistant to p53 induced apoptosis, these results suggest that BCL-2 family members play an essential role in this apoptotic pathway.

Thesis overview

Our lab is interested in the induction of apoptosis by activated oncogenes, namely the E1A viral oncogene. Microarray analysis showed that BIK, a BH3-only protein, is induced by E1A (59). BIK is the founding member of the BH3-only proteins and has been discovered by its ability to bind anti-apoptotic BCL-2 family members (120, 121). In addition to its BH3 domain, it contains a C-

terminal transmembrane domain and two phosphorylation sites at serine 33 and 35 which might enhance its activity (122). BIK binds anti-apoptotic BCL-2 family members but not BAX and BAK (120, 121). The binding to anti-apoptotic BCL-2 proteins through it BH3 is required but not sufficient for its killing activity as aminoacids 122-130 have been reported to be also required, although their function is unknown (123).

Very little information on BIK, other than what was mentioned above, was known at the beginning of this work. Since E1A-induced apoptosis depends on p53 activation, I first set out to confirm that BIK is induced by p53 and started to characterize its activity. The first major surprise came with the realization that BIK is not localized at the mitochondria, as previously described for the other BH3-only proteins, but at the ER. At the time, very little was known about the link between ER and apoptosis. BCL-2 had been shown to localize to the ER in addition of mitochondria but the reason was still obscure. Ca^{2+} signals and the PTP were mostly associated with activities independent of BCL-2 family proteins. One of the only "ER-specific" apoptotic pathways known was that induced by ER stress. ER stress occurs when the capacity of the ER to process proteins in the secretory pathway is exceeded by the amount of protein going in the ER. It results in an increase in unfolded proteins which activates the unfolded protein response (UPR). The cell then shuts down protein synthesis and increases the amount of chaperones in the ER. An incapacity to regulate the problem triggers an apoptotic response that is dependent on the mitochondrial pathway. However, despite its presence at the ER, BIK is unlikely to play a role in this process, as it is not induced upon ER stress (Appendix 1).

The work presented here was thus designed with two goals. The first one was to confirm that BIK induces apoptosis from the ER. The second was to understand the functional role of BIK at the ER. BIK is the only BH3-only protein reported to act on the ER to induce apoptosis and thus its activity was quite puzzling. I first looked at the effect of BIK on mitochondrial morphology, but also looked at the relationship between BIK and BCL-2 at the ER since both proteins are normally found at this location. Therefore, my thesis is divided as follows:

In chapter 2, the ER location of BIK is documented. In addition, I performed *in vivo* and *in vitro* experiments indicating that BIK can induce mitochondria-dependent apoptosis from its location at the ER.

In chapter 3, I describe how, through Ca^{2+}-induced mitochondrial fission, BIK can cooperate with Noxa, another p53-induced BH3-only protein, to induce rapid cytochrome c release and apoptosis.

In chapter 4, BIK-BCL-2 interactions at the ER are studied more closely, revealing that BIK can displace the interaction of BCL-2 with other proteins that are currently being identified.

In chapter 5, I discuss the potential roles of BIK at the ER and their functional significance in the course of apoptosis induction.

The appendix 1 present data supporting a role for the pathways induced by BIK at the ER in the context of p53-induced apoptosis.

Chapter 2: BH-3 Only BIK Functions at the Endoplasmic Reticulum to Stimulate Cytochrome c Release from Mitochondria

The BH3-only protein BIK was identified as a pro-apoptotic molecule interacting with anti-apoptotic BCL-2 homologues. Nothing was known, however, about the regulation of its activity. We found that BIK is upregulated by the E1A oncogene (59) and by p53 (this chapter). Interestingly, while most BH3-only are thought to act directly at the mitochondria to induce cytochrome c release and apoptosis, I found that BIK localizes at the ER. I therefore set to show, through both *in vivo* experiments and *in vitro* reconstitution experiments, that BIK is primary working from the ER to induce mitochondria-dependent apoptosis.

Summary

Stimulation of apoptosis by p53 is accompanied by induction of the BH-3 only pro-apoptotic member of the BCL-2 family, BIK, and ectopic expression of BIK in p53-null cells caused the release of cytochrome c from mitochondria and activation of caspases, dependent on a functional BH-3 domain. A significant fraction of BIK, which contains a predicted transmembrane segment at its COOH-terminus, was found inserted in the endoplasmic reticulum (ER) membrane, with the bulk of the protein facing the cytosol. Restriction of BIK to this membrane by replacing its transmembrane segment with the ER-selective membrane anchor of cytochrome b_5 also retained the cytochrome c release and cell death inducing activity of BIK. Whereas induction of cell death by BIK was strongly inhibited by the caspase inhibitor zVAD-fmk, the inhibitor was without effect on the ability of BIK to stimulate egress of cytochrome c from mitochondria. This zVAD-fmk insensitive pathway for stimulating cytochrome c release from mitochondria by ER BIK was successfully reconstituted *in vitro*, and identified the requirement for components present in the light membrane (ER) and cytosol as necessary for this

activity. Collectively, the results identify BIK as an initiator of cytochrome c release from mitochondria operating from a location at the ER.

Introduction

Apoptosis is a highly regulated mechanism of cell death that is required for normal development and maintenance of tissue homeostasis in multicellular organisms. One of the key events in many types of apoptosis is the release of mitochondrial cytochrome c to the cytosol, along with other pro-apoptotic factors like Smac/Diablo and AIF (124, 125). Cytochrome c, in the presence of dATP/ATP, then triggers the formation of a complex containing procaspase-9 and APAF-1, which leads to activation of caspase-9. Caspase-9 is an initiator caspase that processes effector pro-caspases, resulting in a cascade of proteolytic events and apoptotic death (11).

Diverse upstream death signals appear to be coupled to downstream transformations in mitochondria through the activation of members of a sub-group of the BCL-2 family of proteins, which contain only one of the 4 domains that define BCL-2 proteins, the BH-3 domain (126). One or more of these BH-3 only proteins, including BID, BAD, BIM, Bmf, and others (40, 127), become activated in response to a death signal, which typically causes their translocation to mitochondria. The resulting organelle dysfunction and cytochrome c egress depend on a second pro-apoptotic subgroup of the BCL-2 family located in the mitochondrial outer membrane, the effector molecules BAX and BAK (45, 46). The third subgroup of the BCL-2 family is anti-apoptotic and in addition to the BH-1, -2, and -3 domains found in the pro-apoptotic effectors BAX and BAK, contains a BH-4 domain. When present in the mitochondrial outer membrane in excess, anti-apoptotic BCL-2 members, such as BCL-2 and BCL-X_L, maintain organelle integrity even in the face of sustained death signalling (36, 40). Of note, however, these anti-apoptotic BCL-2 proteins are also found in association with endoplasmic reticulum (ER)[1] and nuclear envelope (35).

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[1] Abbreviations used in this paper. Ad, adenoviral vector; BKA, Bongkrekic acid; ER, endoplasmic reticulum; HA, hemagglutinin; HM, heavy membrane; LM, light

Surveillance of genome integrity is tightly coupled to regulation of apoptosis, primarily through the activity of the p53 tumour suppressor protein. p53 is a transcription factor activated by DNA damage and the expression of certain oncogenes, resulting in either cell cycle arrest or apoptosis (128). While its cell cycle arrest function is well defined, the molecular basis for pro-apoptotic signalling by p53 is only now emerging. It appears to operate by inducing the production of a number of constitutively active pro-apoptotic proteins, each of which is able to independently trigger apoptosis (114, 129). Two of these, Noxa (114) and Puma (112, 113), have been identified as death-inducing BH-3 containing proteins that target and breach mitochondrial integrity. Moreover, the APAF-1/caspase-9 complex has been shown to be necessary for p53 to induce apoptosis, thus implicating cytochrome c release as an important step in the p53 apoptotic pathway (130).

The BH3-only BCL-2 homologue BIK (120, 121) is upregulated in p53-null H1299 cells infected with an adenovirus vector coding for wild-type p53. A significant fraction of cellular BIK localizes to ER membranes where it can induce cytochrome c release from mitochondria. Both *in vitro* reconstitution experiments and engineered targeting of BIK to ER *in vivo*, using the heterologous cytochrome b_5 transmembrane domain, revealed that this process is independent of a direct interaction between BIK protein and mitochondria and does not depend on BAX translocation/insertion in mitochondria. Collectively, our results suggest that BH3-only BIK is capable of initiating cytochrome c release from mitochondria and apoptosis from a location at the ER.

Experimental procedures

Cell culture and infection with adenovirus vectors

H1299 lung carcinoma cells and KB epithelial cells were cultured in α-MEM supplemented with 10% heat-inactivated foetal bovine serum and 100 μg/ml streptomycin and penicillin. Cells were infected at 100 pfu/cell with adenovirus

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membrane; PARP, poly(ADP-ribose) polymerase; PTP, permeability transition pore

vectors expressing either wild-type p53, wild-type BIK tagged with hemagglutinin (HA) epitope at the N-terminus, or a BH3 HA-BIK mutant in which leucine at position 61 was converted to glycine (L61G), as described (131, 132). Cells were collected in phosphate buffered saline (PBS) containing 1.3 mM sodium citrate and 0.6 mM EDTA, centrifuged at 1000 x g for 5 minutes and washed once in PBS. Cell viability was assessed by the ability to exclude trypan blue.

Antibodies and immunoblots

The following antibodies were used: mouse anti-p53 and anti-cytochrome c (PharMingen), anti-PARP (C-II-10) (Biomol), and anti-HA (Babco); rabbit anti-BAX, rabbit anti-cytochrome c and goat anti-BIK (Santa Cruz Biotechnologies); rabbit anti-calnexin and rabbit anti-BiP (gift from J. Bergeron); mouse anti-actin (gift from P. Braun); and chicken antibodies against TOM20 (133) and BAP31 (134). For immunoblot analysis, equal amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (NEN).

Immmunofluorescence

Human KB epithelial cells (80%-90% confluent) were transfected with pcDNA3 vector encoding Flag-BIK or a BIK mutant in which the transmembrane domain (amino acids 135-160) has been replace by the transmembrane domain of rabbit cytochrome b_5 (amino acids 107-134) (Flag-BIKb5TM). After 24 hours, $1x10^4$ cells were centrifuged onto coverslips for 1 min at 2000 x g in a Cyto-Tek centrifuge (Sakura). The recovered cells were then fixed and analyzed by double-label immunofluorescence. Cells were visualized with a Zeiss 510 confocal microscope and images captured and overlaid with the accompanying software.

Cell fractionation

H1299 cells (50% confluence) were either mock infected or infected with Ad vector for the indicated times, harvested, and suspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EGTA) (approx. 10^8 cells/ml). Cells were broken with 25 strokes in a motorized teflon-glass

homogenizer operating at 2000 RPM and the homogenate centrifuged at 1000 x g for 10 minutes to remove nuclei and cell debris (all subsequent steps were for 10 min). The supernatant was centrifuged at 9000 x g, the resulting pellet resuspended in 100 µl HIM and recentrifuged at 9000 x g to give the heavy membrane (HM) fraction enriched in mitochondria. The supernatant from the first 9000 x g centrifugation, designated S9, was centrifuged at 170,000 x g to give the cytosolic supernatant (S100) and light membrane (LM) fractions. In certain instances, the HM and LM fractions were further processed by uniformly resuspending 10 µg of the membrane protein in 150 µl 0.1 M Na_2CO_3, pH 11.5, and incubating on ice for 30 min. Alkaline-insoluble membrane protein was then recovered by centrifugation for 10 min at 170,000 x g. Protein concentrations were determined using the Bio-Rad protein assay.

In vitro cytochrome c release assay

S9 or S100 fractions (described above) were prepared from H1299 cells that were either mock infected (control) or infected with Ad HA-BIK for 14 hours in the presence of 50 µM zVAD-fmk, and designated the "donor fractions". HM on the other hand was prepared only from control H1299 cells (i.e., they lack BIK expression), resuspended at a concentration of 1 µg protein/µl in cMRM (250 mM sucrose, 10 mM HEPES pH 7.5, 1 mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K_2HPO_4), and designated the "acceptor fraction". Alternatively, S100 and HM from mouse liver mitochondria were prepared as described (50). To measure cytochrome c release from mitochondria, S9 fractions containing 35 µg protein in 12.5 µl HIM, or its S100 and LM derivatives, were mixed with 12.5 µl cMRM containing HM (12.5 µg protein) and 50 µM zVAD-fmk, and incubated at 37°C for 30 min. The reaction mixture was then centrifuged at 13,000 x g for 10 minutes and an aliquot of the supernatant subjected to SDS PAGE and immunoblotted with antibody against cytochrome c. For the assays using mouse liver mitochondria and suboptimal amounts pf S9, 15 µg of S9 and 30 µg of mouse S100 were used. Input of mitochondria and recovery of the organelle in the pellet was typically monitored by blotting with antibody against the human mitochondrial protein import receptor located in the outer membrane, TOM20.

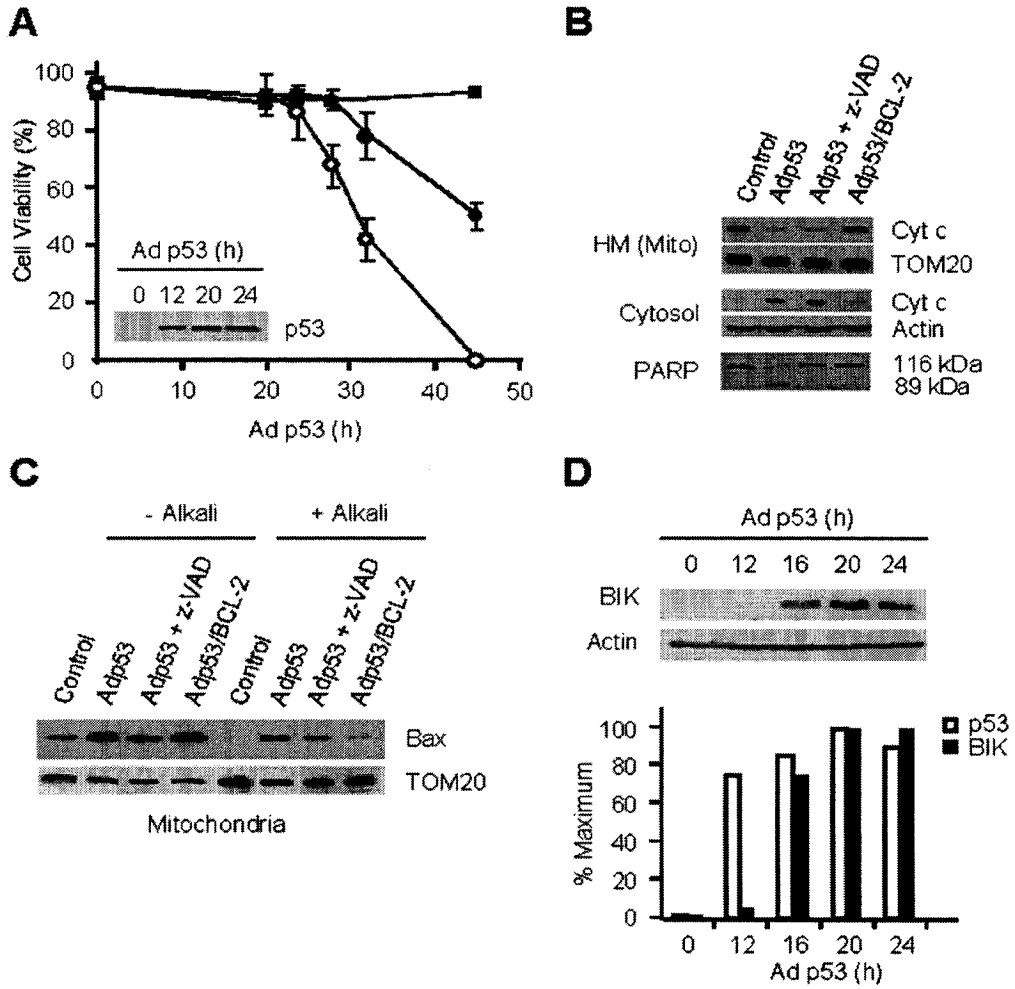


Figure 2.1 ***Induction of apoptosis by p53 in p53-null H1299 cells.*** (A), Time course of cell death. Cells were infected with a control adenovirus vector (Ad LacZ) (filled squares) or Ad p53 in the presence (filled circles) or absence (open circles) of 50 μM zVAD-fmk, and cell viability measured as the % of cells (± standard deviations) that excluded trypan blue. *Insert.* At the indicated times, aliquots of whole cell lysates containing equivalent protein were subjected to SDS PAGE and immunoblotted with antibody against p53. (B), Cytochrome c release from mitochondria and caspase activation following p53 induction. Cells were treated with Ad p53 for 20 hours as indicated, fractionated into heavy membranes (HM, enriched in mitochondria) and S100 (cytosol), subjected to SDS PAGE, and blots probed with antibody against cytochrome c (Cyt c). For comparisons, the HM fractions were also probed with antibody against TOM20 and the cytosolic fractions with antibody against γ-actin, which served as gel loading controls for the two fractions, respectively. Additionally, whole cell lysates were analyzed by immunoblotting with antibody against PARP, with the full-length protein (116 kDa) and 89 kDa apoptotic fragment indicated. (C), Insertion of BAX into mitochondrial membrane. Cells were treated and fractionated as in B. Mitochondria were analyzed by immunoblotting with antibodies against BAX and TOM20 either directly (- Alkali, lanes 1-4) or after extraction with 0.1 M Na_2CO_3, pH 11.5 (+ Alkali, lanes 5-8). (D), Induction of BIK by p53. As in A except that cell lysates were probed with antibodies against BIK and γ-actin (upper panel). In the lower panel, relative levels of p53 and BIK were determined following infection of cells with Ad p53 by quantifying immunoblot signals using a Power Macintosh 7200/120 and NIH Image v.1.61 image analysis software. Representative results are presented.

Results

Delivery of an adenoviral vector expressing the wild-type p53 tumour suppressor protein (Ad p53) to the p53-null H1299 lung carcinoma cell line resulted in induction of p53 protein within 12 hours, followed by cell death that was partially inhibited by the general caspase inhibitor zVAD-fmk (Figure 2.1A). Cytochrome c was released from mitochondria and caspases were activated, as reflected by the cleavage of poly(ADP-ribose) polymerase (PARP) (Figure 2.1B). Although zVAD-fmk was able to completely block PARP cleavage, it did not have any effect on the release of cytochrome c, suggesting that the molecular events leading from p53 activation to cytochrome c release are not strongly dependent on zVAD-fmk-sensitive caspases. On the other hand, BCL-2 inhibited both cytochrome c release from mitochondria and PARP cleavage (Figure 2.1B).

The pro-apoptotic BCL-2 homologue BAX is typically found loosely associated with heavy membranes (HM) enriched in mitochondria, in addition to its cytosolic localization in untreated cells. Upon an apoptotic stimulus, however, it becomes integrated into the outer mitochondrial membrane and resistant to alkali extraction (54). As it was seen for cytochrome c release, acquisition of alkaline-resistance of BAX in response to Ad p53 was insensitive to zVAD-fmk but was inhibited by BCL-2 (Figure 2.1C).

In addition to the activation of BAX, p53 is believed to induce apoptosis by upregulating multiple pro-apoptotic proteins, including members of the BH3-only class of the BCL-2 family, such as Noxa (114) and Puma (112, 113). The BH3 only protein BIK (120, 121) is another potential p53 target as it was induced by the ectopic expression of p53 in H1299 cells, while being undetectable in control cells (Figure 2.1D). Induction of BIK by Ad p53 occurred before the cells showed overt signs of loss of viability (starting at 24 h), as assessed by the exclusion of trypan blue (Figure 2.1A and D), with the kinetics of BIK induction paralleling that of cytochrome c release and the acquisition of BAX alkaline resistance (Figure 1B and C and data not shown).

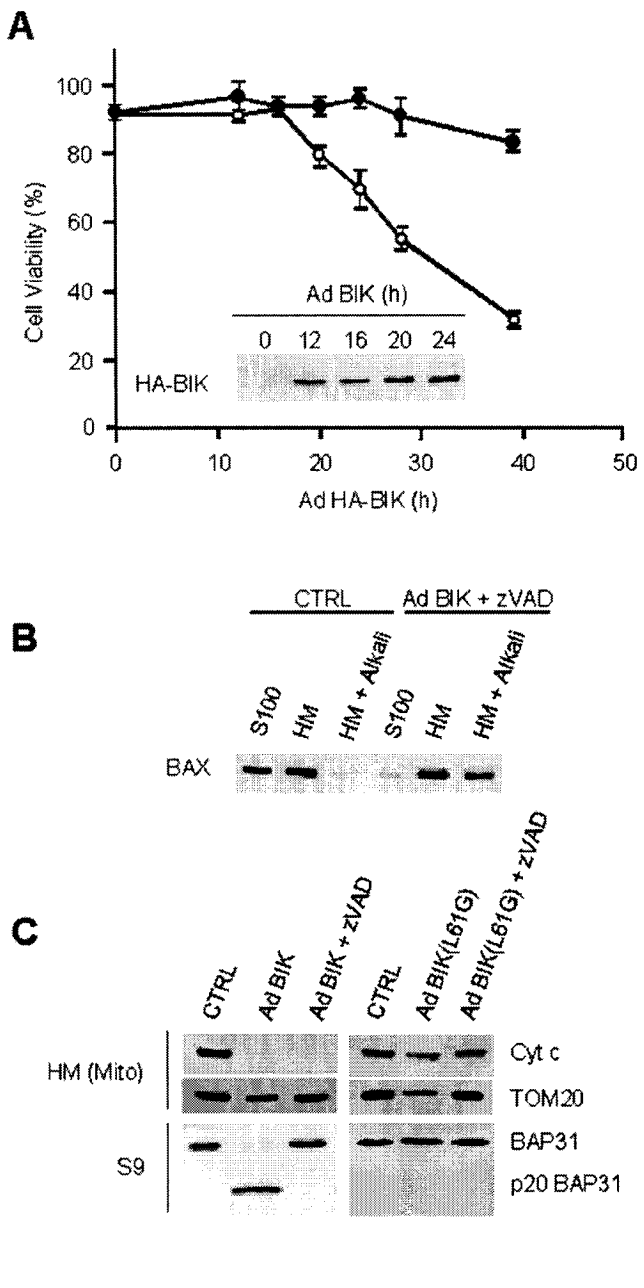


Figure 2.2 ***BIK induces BAX insertion into mitochondria, release of cytochrome c, and cell death in p53-null H1299 cells.*** (A), Time course of cell death. Cells were infected with Ad HA-BIK in the presence (Filled circles) or absence (open circles) of 50 μM zVAD-fmk, and cell viability measured as the % of cells (± standard deviations) that excluded trypan blue. *Insert.* At the indicated times, aliquots of whole cell lysates containing equivalent protein were subjected to SDS PAGE and immunoblotted with antibody against HA. (B), H1299 cells were mock-infected (CTRL) or infected with Ad HA-BIK for 12 h in the presence of 50 μM zVAD-fmk, and fractions corresponding to S100 (cytosol), HM (enriched in mitochondria), and alkaline-extracted HM were prepared (see Fig. 1). Aliquots containing equivalent protein were immunoblotted with antibody against BAX. (C), H1299 cells were mock-infected (CTRL) or infected for 12 h with Ad HA-BIK or Ad HA-BIK(L61G) in the presence or absence of 50 μM zVAD-fmk. HM (enriched in mitochondria) and S9 (containing LM and cytosol) fractions were recovered and analyzed by immmunoblotting with antibodies against cytochrome c (Cyt c), TOM20, and BAP31 as indicated. p20 BAP31 is the 20 kDa caspase cleavage product of BAP31 (28 kDa).

BIK induces cytochrome c release from mitochondria and caspase activation

In order to study directly BIK-induced apoptosis, an adenovirus expressing wild-type HA-tagged BIK was employed. Induction of BIK in p53-null H 1299 c ells using this system resulted in cell killing that was inhibited by zVAD-fmk (Figure 2.2A), indicating the requirement for caspases. BIK also triggered the insertion of BAX into mitochondrial membrane (Figure 2.2B). This was accompanied by loss of cytochrome c from mitochondria within 14 hours of treatment and activation of

caspases as judged by the cleavage of the caspase target BAP31 (134) (Fig. 2.2C). While zVAD-fmk prevented cell death (Figure 2.2A) and caspase cleavage of BAP31 (Figure 2.2C), it had little effect on membrane insertion of BAX and cytochrome c release from mitochondria, suggesting that, like p53, BIK-induced mitochondrial dysfunction is likely to be independent of zVAD-fmk-sensitive caspases. In addition, BIK activity required a functional BH3 domain, since a BIK mutant in which the conserved leucine 61 in the BH3 domain was mutated to a glycine (BIK(L61G)) did not cause cell death (not shown), cytochrome c release, or caspase cleavage of BAP31 (Figure 2.2C). Mutant BIK expression was higher that of wild-type BIK (not shown).

A significant amount of BIK localizes to the ER

In order to study the cellular localization of BIK, KB epithelial cells were transiently transfected with Flag-tagged wild-type BIK and examined by immunofluorescence confocal microscopy. Flag-BIK showed extensive colocalization with the ER marker calnexin, and this reticular network mostly comprised regions within the cell that did not include the mitochondrial outer membrane marker TOM20 (representative images are shown in Figure 2.3A). Similarly, fractionation of H1299 cells infected with Ad HA-BIK revealed a co-distribution of HA-BIK and calnexin in the light membrane (LM) fraction. HA-BIK was also recovered in the HM fraction containing mitochondria, as judged by the presence of TOM20, but this fraction also contained the ER protein calnexin (Figure 2.3B). Similar results were obtained with endogenous BIK following infection of H1299 cells with Ad p53 (data not shown). LM-associated BIK was resistant to alkali extraction but sensitive to proteinase K digestion (Figure 2.3C), suggesting that BIK is integrated in the ER membrane and facing the cytosolic side. As controls, transmembrane calnexin was sensitive to proteinase K (employing an antibody raised against its cytosolic tail) while the lumenal chaperone BiP was resistant, and only BiP was extracted by alkali (Figure 2.3C).

These experiments suggested that BIK might induce cytochrome c release and cell death from an ER location, although partial association of BIK with the mitochondria cannot be ruled out. Thus, to address the contribution of ER-

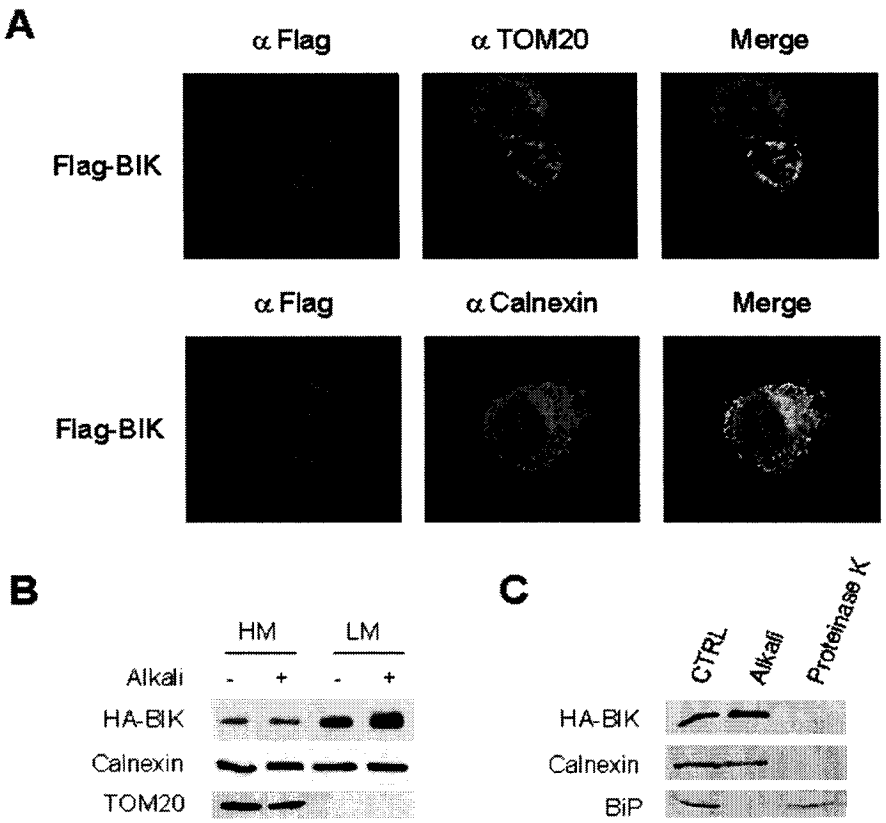


Figure 2.3 ***Presence of HA-BIK in the endoplasmic reticulum.*** **(A), KB epithelial cells were transfected with plasmid expressing Flag-BIK and recovered on coverslips by centrifugation after 24 h. The cells were double stained with anti-Flag antibody (Alexa 594 [red]) and either anti-Calnexin (ER marker) or anti-TOM20 (mitochondrial outer membrane marker) antibodies (Alexa 488 [green]), and images of the same cell visualized in the red, green, or merged (yellow) channels. Representative images are shown. (B), Following infection of H1299 cells with Ad HA-BIK for 20 h, the indicated cell fractions were generated and either left untreated or subjected to alkali extraction. Aliquots from equivalent number of cells were analyzed by immunoblotting with antibody against HA, calnexin, and TOM20. (C), S9 extracts from Ad HA-BIK-infected H1299 cells were either left untreated, subjected to alkali extraction or to proteinase K for 30 min at 4?C, after which LM were spun down at 170 000 x g for 10 minutes. The resulting pellets were analyzed by immunoblotting with antibodies against HA, calnexin and the ER lumenal chaperone BiP.**

localized BIK to cytochrome c release and cell death more fully, we generated a mutant BIK in which its C-terminal transmembrane domain was replaced by the C-terminal transmembrane segment of cytochrome b5 (Flag-BIK-b5TM), a sequence previously shown to selectively target fusion proteins to the ER (135, 136). The intracellular localization of this mutant was first studied by immunofluorescence confocal microscopy following transient transfection into KB cells. As shown in the representative images in Figure 2.4A, Flag-BIK-b5TM strongly co-localized with ER calnexin. Like Flag-BIK, Flag-BIK-b5TM was able

to cause cytochrome c release from mitochondria in the presence of zVAD-fmk (Figure 2.4B). Both proteins also induced cell death, as measured by a luciferase reporter essay (Figure 2.4C) or by visual examination of cells co-transfected with vector expressing green fluorescent protein (not shown). That Flag-BIK-b5TM triggered cytochrome c release from mitochondria and cell death argues that BIK can function through its location in the ER.

In vitro release of cytochrome c from mitochondria lacking BIK by LM containing HA-BIK

To establish that BIK present in the ER can indeed stimulate cytochrome c release from mitochondria, the system was reconstituted *in vitro* (Figure 2.5A). The assay comprised a donor and an acceptor fraction. The donor is a S9 extract from Ad HA-BIK-infected H1299 cells which contains LM and HA-BIK but that is free of mitochondria (as judged by the absence of TOM20) (Figure 2.5B). The acceptor fraction is a HM fraction from uninfected cells that is enriched in mitochondria but contains no HA-BIK (Figure 2.5B) nor endogenous BIK (Figure 2.1D). To minimize the influence of caspases, zVAD-fmk was both provided to the cells during Ad HA-BIK infection and included in the *in vitro* assays. The influence of the S9 or its derived S100 and LM components on recipient HM was determined by incubating donor fractions with acceptor HM *in vitro* for 30 min at 37°C, recovering the HM by centrifugation at 13,000 x g, and measuring release of cytochrome c into the supernatant by immunoblotting. In all cases, equivalent amounts of S9 protein (35 μg) were added to the reactions.

As shown in Figure 2.5C, donor S9 from Ad HA-BIK-infected cells, but not from control cells, induced the release of cytochrome c from mitochondria. This effect was dependent on the presence of the LM as their removal prevented cytochrome c release (Figure 2.5C, compare S100 and S9). Cytochrome c release that was induced by LM-associated HA-BIK was also dependent on a functional BH3 as the L61G mutant was inactive (Figure 2.5D). Of note, there was no detectable presence of HA-BIK recovered with the HM after incubation with donor HA-BIK S9 (Figure 2.5E) whereas most of the TOM20 was recovered in this fraction. This

indicates that stimulation of cytochrome c release from mitochondria by S9 HA-BIK did not occur because HA-BIK translocated from ER to mitochondria.

Efficient cytochrome c release by Ad HA-BIK in vitro is blocked by mitochondrial BCL-2, and is independent of PTP, Ca^{2+} and Mg^{2+}

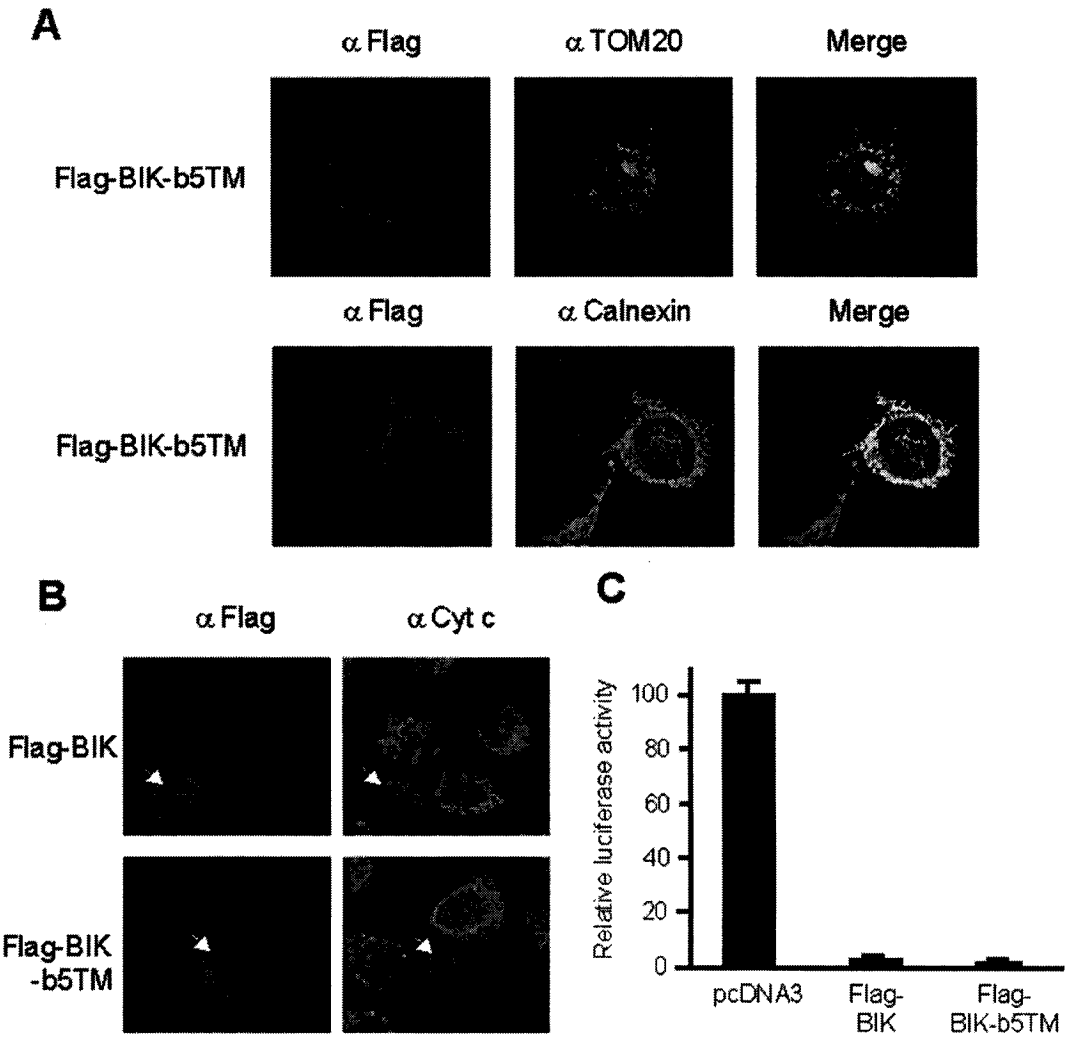


Figure 2.4 ***Endoplasmic reticulum-targeted BIK causes cytochrome c release from mitochondria and cell death.*** (A), Cells were transfected with Flag-BIKb5TM and analyzed by immunofluorescence as in Fig. 3A. (B), Wild-type Flag-BIK and Flag-BIK-b5TM were transfected as in A and double stained with anti-Flag antibody (Alexa 594 [red]) and anti-cytochrome c antibody (Alexa 488 [green]). Cells were visualized in the red and green channels by fluorescence microscopy. *Arrows*, denote cells expressing Flag-BIK or Flag-BIK-b5TM. Representative images are shown. (C), H1299 cells were transfected with reporter plasmid expressing luciferase together with pcDNA3 vector alone or expressing either Flag-BIK or Flag-BIK-b5TM. After 24 h, cell lysates were prepared and luciferase activity determined on aliquots containing equivalent protein, and expressed relative to the maximum activity obtained. Shown are the results of 3 independent determinations ± standard deviations.

Although BIK can interact with anti-apoptotic members of the BCL-2 family (120, 121), the *in vitro* reconstitution system afforded the opportunity to investigate the influence of BCL-2 operating downstream of BIK. This was addressed in the *in vitro* system by using HM from cells that over-express BCL-2 (131). As shown in Figure 2.6A, mitochondrial BCL-2 efficiently prevented cytochrome c release by HA-BIK S9. The influence of BCL-2 on the integrity of mitochondrial outer membrane and cytochrome c release has been suggested to be related to its effects on mitochondrial permeability transition pore (PTP) (20, 29). The involvement of PTP in the *in vitro* BIK-induced cytochrome c release was thus tested using the PTP inhibitor bongkrekic acid (BKA). As shown in Figure 2.6B, 100 μM BKA did not block BIK-induced cytochrome c release but it inhibited cytochrome c release by a known PTP activator, Ca^{2+}, indicating that the PTP does not play a major role in LM BIK-induced cytochrome c release.

ER membranes have been shown to influence mitochondria by controlling the intracellular stores of divalent cations, mainly Ca^{2+} (71). While a role of Ca^{2+} is unlikely in our *in vitro* system as 1 mM EGTA was present in the incubation buffer and Ca^{2+}-sensitive PTP does not appear to contribute to cytochrome c release (Figure 2.6B), other cations such as Mg^{2+} could be important (137). However, 5 mM EDTA did not modulate the cytochrome c release activity of the HA-BIK S9 donor fraction (Figure 2.6A). Thus, the BIK-induced cytochrome c release pathway observed here is unlikely to be mediated by an effect on the levels of free Ca^{2+} or Mg^{2+}.

LM BIK-induced cytochrome c release requires the LM in addition to a cytosolic factor independent of BAX

Since infection of H1299 cells with Ad HA-BIK promotes BAX insertion into mitochondrial membrane (Figure 2.2B) and since regulated BAX insertion into mitochondrial membrane is a potential effector of BIK-induced cytochrome c release (45, 46), its role in the *in vitro* system was investigated. As shown in Figure 2.7A, S9 extract from Ad-HA-BIK infected cells but not from control cells caused BAX to become alkali resistant. As S9 Ad BIK contains very little BAX (Figure 2.7B, Input H1299 S9 Ad BIK), the origin of alkali resistant BAX is

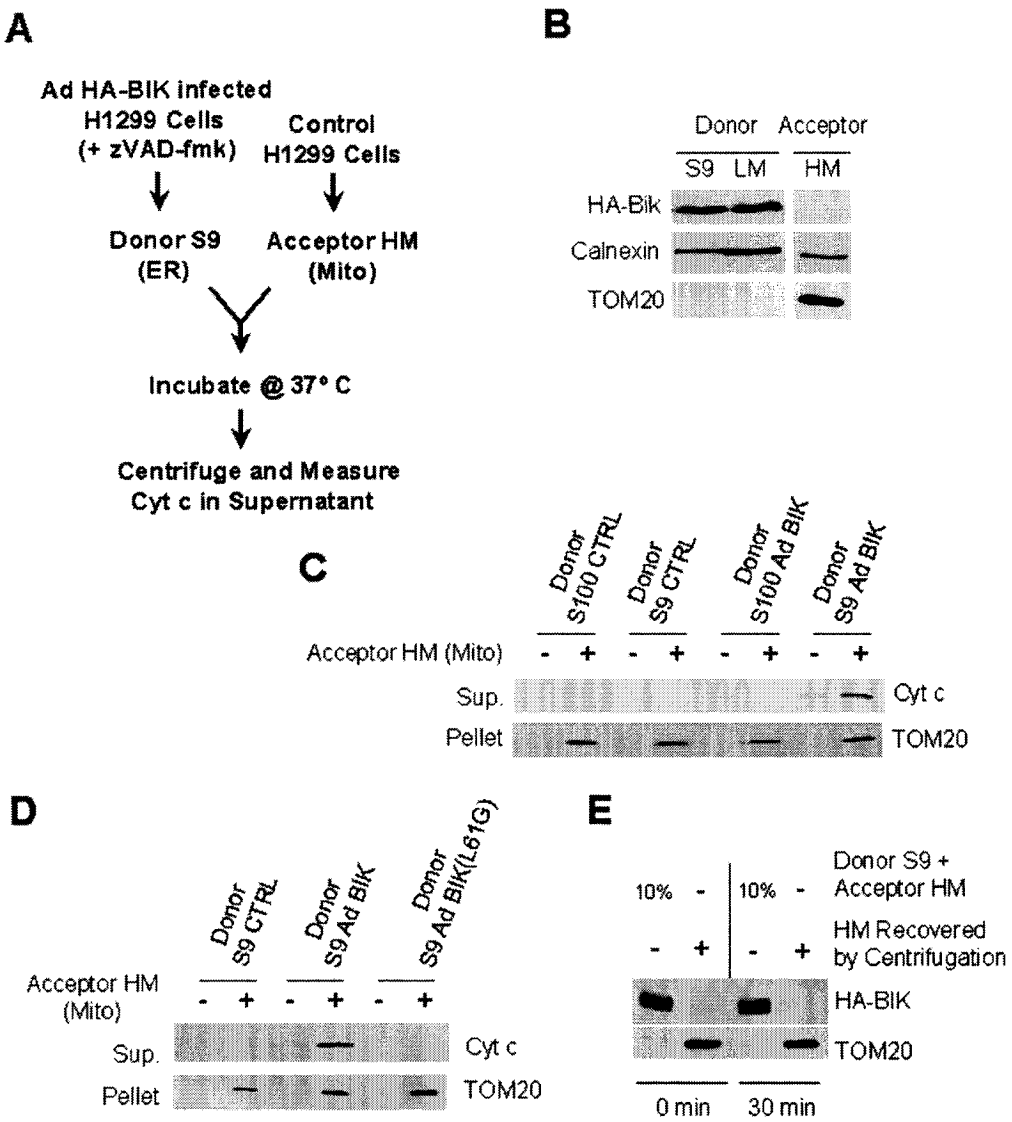


Figure 2.5 ***Donor S9 HA-BIK stimulates BAX membrane insertion and mitochondrial release of cytochrome c in an acceptor HM fraction lacking BIK.*** (A) The assay scheme. Donor S9 fraction was prepared from H1299 cells infected with Ad HA-BIK for 12 hours in the presence of 50 μM zVAD-fmk. Centrifugation of S9 for 10 min at 170 000 x g yielded the S100 (supernatant) and LM (pellet) fractions. LM was resuspended in a volume of HIM buffer equal to that of S100. Acceptor HM (enriched in mitochondria) was prepared from uninfected H1299 cells. (B), Donor S9 and LM fractions and acceptor HM were probed with antibody against HA, Calnexin, and TOM20. (C) The indicated donor fractions were prepared from mock infected (CTRL) or Ad HA-BIK infected cells and incubated at 37?C for 30 min with (+) or without (-) acceptor HM in the presence of 50 μM zVAD-fmk. At the end of the incubation, reaction mixtures were centrifuged at 13,000 x g to yield supernatants and pellets, which were probed with antibodies against cytochrome c (supernatant) and TOM20 (pellet). (D) As in C except that donor fractions were prepared from cells infected with Ad vectors expressing either wt HA-BIK or mutant HA-BIK(L61G). (E) Donor S9 from Ad HA-BIK infected cells was combined with acceptor HM and 10% removed and dissolved in SDS sample buffer (input). The remainder was incubated for 0 or 30 min at 37° and the HM recovered. The resulting pellet and 10% input were immunoblotted with antibodies against HA and TOM20.

presumably that which is associated with the acceptor HM (see Figure 2.2B, lane 2). To better assess the contribution of BAX, therefore, we prepared HM from BAX-null liver. Both $BAX^{-/-}$ and $BAX^{+/-}$ acceptor HM could support cytochrome c release by H1299 S9 Ad BIK (data not shown). In addition, when using limiting concentration of the donor S9 that marginally cause cytochrome c release by itself, cytochrome c release was achieved by adding S100 from either $BAX^{+/-}$ or $BAX^{-/-}$ mouse liver (Figure 2.7B), whereas the S100 on their own failed to induce cytochrome c release (data not shown). Under all conditions tested, there was no translocation of BAX to mitochondria (Figure 2.7B). All together, these results indicate that BIK requires the presence of a constitutive cytosolic component to induce cytochrome c release from mitochondria, which is not BAX.

Since the depletion of LM from H1299 S9 Ad BIK abrogated its capacity to induce cytochrome c release (Figure 2.5C), LM is also a required constituent. In contrast, LM on its own failed to induce cytochrome c release (Figure 2.7C). In addition, no cytochrome c release was observed when the donor S9 fraction was preincubated for 30 minutes at 37°C, after which the LM were spun down and the resulting supernatant was used as the donor in the cytochrome c release assay (Figure 2.7C), indicating that a sustained presence of the LM is required. Thus, a complex signalling pathway is initiated by BIK, requiring both LM and cytosolic constituents.

Discussion

Recent evidence suggests that BH-3 only BCL-2 homologues induce apoptosis by binding to mitochondria and causing cytochrome c release, dependent on the effector proteins BAX and BAK (45, 46). A number of these BH-3 only members, like BID and BAD, become activated in response to death signals through structural changes to pre-existing inactive conformers (36). In contrast, p53 stimulates the production of several constitutively active BH-3 only proteins, including BIK, Puma, and Noxa, each of which can autonomously induce mitochondrial dysfunction and cell death. While Noxa and Puma have been reported to influence mitochondrial integrity directly (112-114), we show here

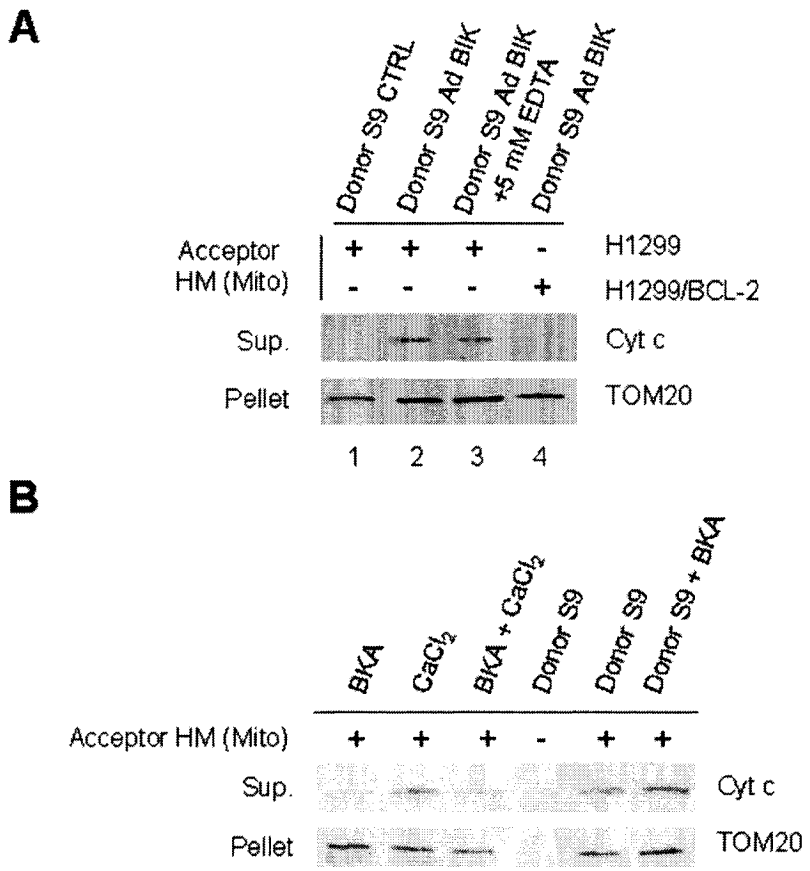


Figure 2.6 ***LM-BIK-induced cytochrome c release in vitro is blocked by BCL-2 but is independent of the PTP.*** (A) Donor S9 HA-BIK does not stimulate mitochondrial release of cytochrome c in HM from H1299 cells over-expressing BCL-2. Donor fractions were prepared from Ad HA-BIK infected cells and incubated with acceptor HM obtained from H1299 cells either lacking or stably expressing ectopic BCL-2 (20), in the presence or absence of 5 mM EDTA. Reaction mixtures were then separated into pellet and supernatant and probed with antibody against TOM20 or cytochrome c, respectively. (B) The PTP inhibitor BKA does not inhibit LM-BIK-induced cytochrome c release. Donor HA-BIK S9 were incubated with acceptor HM in absence or presence of 100 μM BKA and processed as in A. As a control, acceptor HM was incubated in presence of 200 μM $CaCl_2$, with or without BKA.

that BIK can stimulate mitochondrial release of cytochrome c from a location at the ER. This is the first demonstration of a canonical BH-3 only member of the BCL-2 family initiating an apoptotic signalling pathway from this organelle.

A significant proportion of both overexpressed and endogenous BIK was found to co-localize with the ER marker calnexin, both by immunofluorescence in KB epithelial cells and by biochemical fractionation in H1299 cells. Although a second pathway involving mitochondrial BIK cannot be excluded, results from

both *in vitro* reconstitution and the demonstration that Flag-BIK-b5TM can induce cytochrome c release from mitochondria *in vivo* and cell death argues that ER-localized BIK is functional. Of note, however, we have found that APAF-$1^{-/-}$ cells are resistant to BIK-induced caspase activation and cell death but that BIK still induces cytochrome c release from their mitochondria (not shown). This result confirms that BIK operates upstream of cytochrome c release in an obligate Apaf-1-mediated death pathway, as previously suggested by the use of a caspase-9 dominant negative (138).

The relationship between induction of ER (LM)-localized BIK and release of cytochrome c from mitochondria was studied in an *in vitro* system in which a S9 fraction from Ad HA-BIK H1299 cells was incubated with an HM fraction from control cells lacking BIK. It can be concluded from these experiments that ER-localized BIK triggers a cytochrome c releasing activity that is dependent on the BIK BH3 domain, as well as on the presence of both LM and cytosol. In this *in vitro* system, ER BIK did not dissociate from the LM to impose its activity by direct binding to mitochondria. In addition, cytochrome c release was not influenced by the PTP inhibitor BKA and was independent of BAX translocation/insertion into mitochondrial membrane. Although regulated targeting of BAX is one way in which mitochondrial dysfunction can be coupled to an upstream death signal, the lack of dependence may reflect the redundancy provided by the BAX homologue BAK, which is constitutively present in the mitochondrial outer membrane (45, 50). Collectively, however, our results suggest a complex pathway initiated by BIK, in which BIK regulates mitochondrial dysfunction through both ER and cytosolic factors independent of cytosolic BAX.

BIK was discovered through a search for proteins that interact with anti-apoptotic BCL-2 proteins and was subsequently shown to readily co-immunoprecipitate with BCL-2 and BCL-X_L (120, 121, 123). These anti-apoptotic BCL-2 homologues have been shown to be associated with ER and nuclear membranes in addition to their mitochondrial location (35). The ratio of pro-apoptotic BIK and anti-apoptotic BCL-2 homologues in the ER, therefore, may influence the ER

pathway that regulates mitochondrial integrity. Although a pathway involving Ca^{2+} is an obvious candidate, neither this cation nor Mg^{2+} appears to be involved in the pathway described here. Of particular note, however, we also found that excess BCL-2 in the acceptor HM blocked cytochrome c release after stimulation with S9 Ad BIK, indicating that BCL-2 can also function downstream on the BIK pathway.

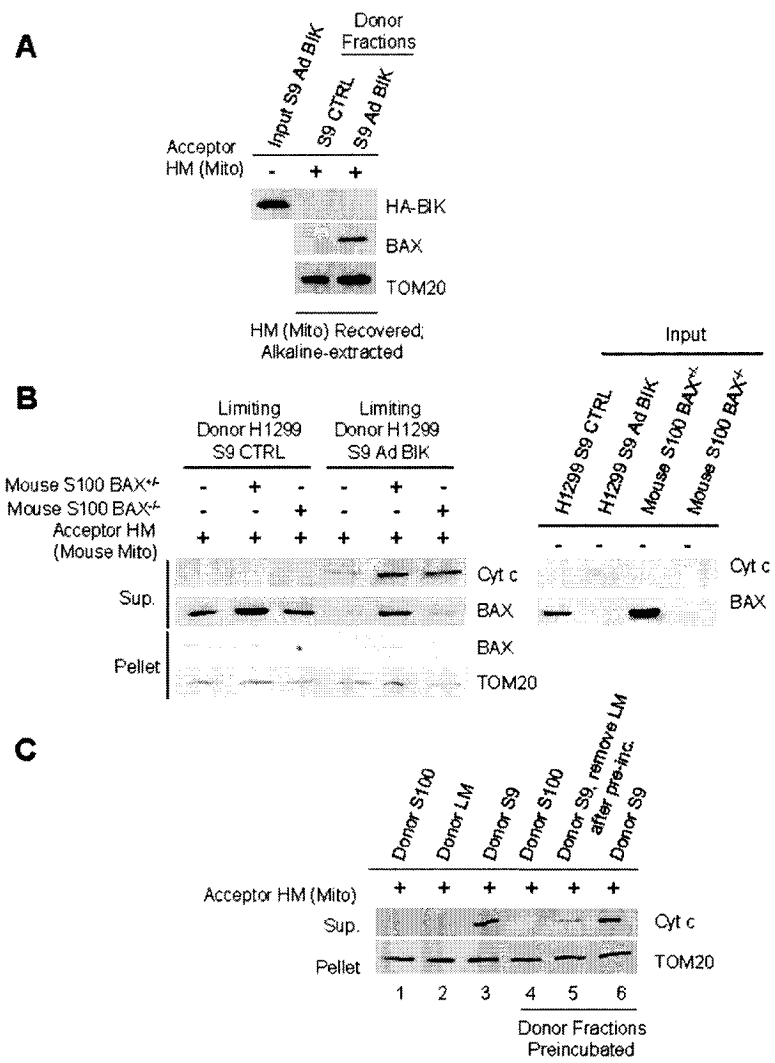


Figure 2.7 ***LM-BIK-induced cytochrome c release requires a cytosolic factor that is independent of BAX.*** (A) Acceptor HM was incubated at 37?C for 30 min with the indicated donor fractions, the HM recovered by centrifugation at the end of the incubation, subjected to alkaline extraction, and analyzed by immunoblotting with antibodies against HA, BAX, and TOM20. An aliquot of the input S9 Ad HA-BIK fraction was immunoblotted with anti-HA (lane 1). (B) Acceptor HM from $BAX^{-/-}$ or $BAX^{+/-}$ mouse liver were incubated with sub-optimal amounts of CTRL or BIK donor S9 in absence or presence of S100 prepared from either $BAX^{-/-}$ or $BAX^{+/-}$ mouse liver and processed as in Fig. 5. (C) Acceptor HM was incubated with the indicated donor fractions (lanes 1-3), the HM subsequently recovered by centrifugation, and the resulting pellets and supernatants probed with antibodies against TOM20 and cytochrome c, respectively. Alternatively, the donor fractions were first pre-incubated in the absence of HM for 30 min at 37°(lanes 4-6) and in the case of S9, the LM was or was not removed by centrifugation at 170,000 x g for 10 min. These pre-incubated donor fractions were then analyzed for their ability to release cytochrome c from acceptor HM as in lanes 1-3.

In conclusion, our data identifies BIK as initiating a pathway from its location in the ER that stimulates cytochrome c release from mitochondria and that would be activated upon the induction of BIK protein by p53. This p53 pathway is distinct from other p53-induced targets such as the mitochondrial BH3-only proteins

Noxa and Puma, suggesting yet another level at which p53-dependent apoptosis is controlled.

Acknowledgements

This work was supported by grants from National Cancer Institute of Canada and the Canadian Institute of Health Research. MG holds studentships from CIHR and the Fond pour la Recherche en Santé du Québec. JPM holds a studentship form CIHR. We are thankful to Richard Marcellus for the Ad HA-BIK vector constructs, to Ruth Slack for the BAX knockout animals and to John Bergeron and Peter Braun for providing antibodies.

Chapter 3: Endoplasmic reticulum BIK initiates DRP1-dependent mitochondrial dynamics and cristae remodeling. cooperation with NOXA to stimulate cytochrome c release to the cytosol

At the time of publishing the discovery that BIK induces apoptosis from an ER location, very little was known on apoptotic pathways emanating from the ER. BIK thus provided a specific tool to assess the function of pro-apoptotic proteins at this location. As the ER is a major site for Ca^{2+} regulation and that Ca^{2+} plays a role in regulating cell death, I first looked at the effect of BIK induction on Ca^{2+} signals and their relationship with mitochondrial fission which was emerging as an important step in the induction of apoptosis. This Chapter thus examine the pro-apoptotic functions of BIK at the ER early after its induction.

Abstract

BIK, a BH3-only member of the BCL-2 family, is induced by p53 and elicits pro-apoptotic stimuli from its location in the endoplasmic reticulum (ER) membrane. Early effects of BIK include the release of Ca^{2+} from ER stores and DRP1-mediated fission of mitochondria resulting from uptake of Ca^{2+} into the organelle, but little release of cytochrome c to the cytosol. These fragmented mitochondria are dynamic structures, undergoing repeated cycles of expansion and contraction. Additionally, the DRP1-dependent events are associated with remodeling of cristae and an expansion of the intermembrane compartment. Compared to mitochondria from unstimulated cells, mitochondria from BIK-stimulated cells exhibit enhanced release of cytochrome c when the outer membrane is physically breached with digitonin. Another p53-induced BH3-only protein, NOXA, does not cause mitochondrial fission or cytochrome c release. However, NOXA strongly cooperates with BIK to elicit conformational changes in mitochondrial BAX, robust DRP1-dependent release of cytochrome c, and activation of caspases. We propose a model in which BIK initiates DRP1-dependent dynamic

structural transformations in mitochondria and mobilization of internal cytochrome c stores, permitting cooperation with a stimulus provided by another BH3-only protein to induce rapid apoptotic cell death.

Introduction

Elimination of cells that are either damaged or represent a potential threat to an organism, such as pre-cancerous cells, is achieved through a programmed form of cell death called apoptosis. In many cases, the apoptotic stress pathways in these cells are coupled to stimulation of an intermediate, p53. Elevated p53 then turns on a transcription program that activates numerous genes that encode proteins capable of inducing the apoptotic machinery (reviewed in 4). Among these gene products are regulators of the mitochondrial death pathway, including members of the pro-apoptotic BH3-only sub-group of the BCL-2 protein family and Apaf-1, a cytosolic adaptor protein that interacts with cytochrome c following its release from mitochondria, which in turn leads to recruitment and activation of caspase-9 (7). To date, four BH3-only proteins – NOXA (114, 118, 119), PUMA (112, 113, 116), BID (139), and BIK (59, 140) – are known to be induced in response to p53 activation. BH3-only BCL-2 members have the potential to gain direct access to the core mitochondrial apoptosis machinery because, in their active conformation, they regulate the ability of the multi-BH domain members, BAX and BAK, to oligomerize in the mitochondrial outer membrane and release intermembrane proteins, including cytochrome c, to the cytosol (46, 50). Anti-apoptotic BCL-2 proteins, on the other hand, can form dimers with the activated conformers of pro-apoptotic members and block this progression of events at the mitochondrial surface (49, 62). These steps have all been reconstituted *in vitro* utilizing either isolated mitochondria (50) or proteoliposomes of defined compostion (141), arguing that BCL-2 family proteins are responsible for executing and regulating the critical path leading to mitochondrial apoptosis. An important question, however, arises from the finding that p53 activates multiple BH3-only proteins, each of which has been shown on its own to be an initiator of mitochondrial apoptosis when expressed ectopically for sufficient periods of time in p53-

deficient cells (112-114, 120). The fact, however, that selective interference with any one of them retards p53-induced cell death (114, 116, 139) argues that these multiple regulators may reflect cooperation, rather than redundancy, in the pathways that control mitochondrial apoptosis in intact cells.

Convergence of cooperating pathways on mitochondria was first demonstrated by Youle and colleagues who found that recruitment and activation of dynamin-related protein-1 (DRP1) at discrete sites on the mitochondrial tubular network in intact cells was requisite for egress of cytochrome c to the cytosol and apoptosis in intact cells (86). DRP1 is a GTPase that causes scission of the outer mitochondrial outer membrane, resulting in fission of the mitochondrial tubules into punctiform fragments (reviewed in (89, 90)). DRP1-mediated mitochondrial fission can be induced by the release of Ca^{2+} from endoplasmic reticulum (ER) stores, which in one case is initiated by caspase-8 cleavage of BAP31, an integral protein of the ER membrane (87). These calcium and DRP1-dependent events initiated by the p20 cleavage product of BAP31 cooperate with other caspase-8-initiated pathways, notably conversion of BID to its active conformer, tBID, to generate robust release of cytochrome c to the cytosol. The mechanism underlying the Ca^{2+}- and DRP-1-dependent sensitization of mitochondria to direct pro-apoptotic insults, however, is poorly understood (92).

Given the probable universality of ER Ca^{2+} signaling during apoptosis (8, 68, 142), it is likely that such a 2-hit mechanism for mitochondrial apoptosis in intact cells extends to other death pathways, and involves regulators at the ER and mitochondria commensurate with the death stimulus in question. In the case of targets of p53 induction, we demonstrate here that Ca^{2+}-mediated fragmentation of mitochondria and remodeling of inner membrane cristae is initiated by BIK at the ER. Opening of cristae in response to BIK correlates with mobilization of intraorganellar cytochrome c stores, permitting BIK to cooperate with NOXA to stimulate mitochondrial BAX, cause cytochrome c release from the organelle, and activate effector caspases. The underlying structural changes in mitochondria that arise in response to BIK expression suggest a mechanism for sensitizing the organelle to apoptotic release of cytochrome c and induction of apoptosis.

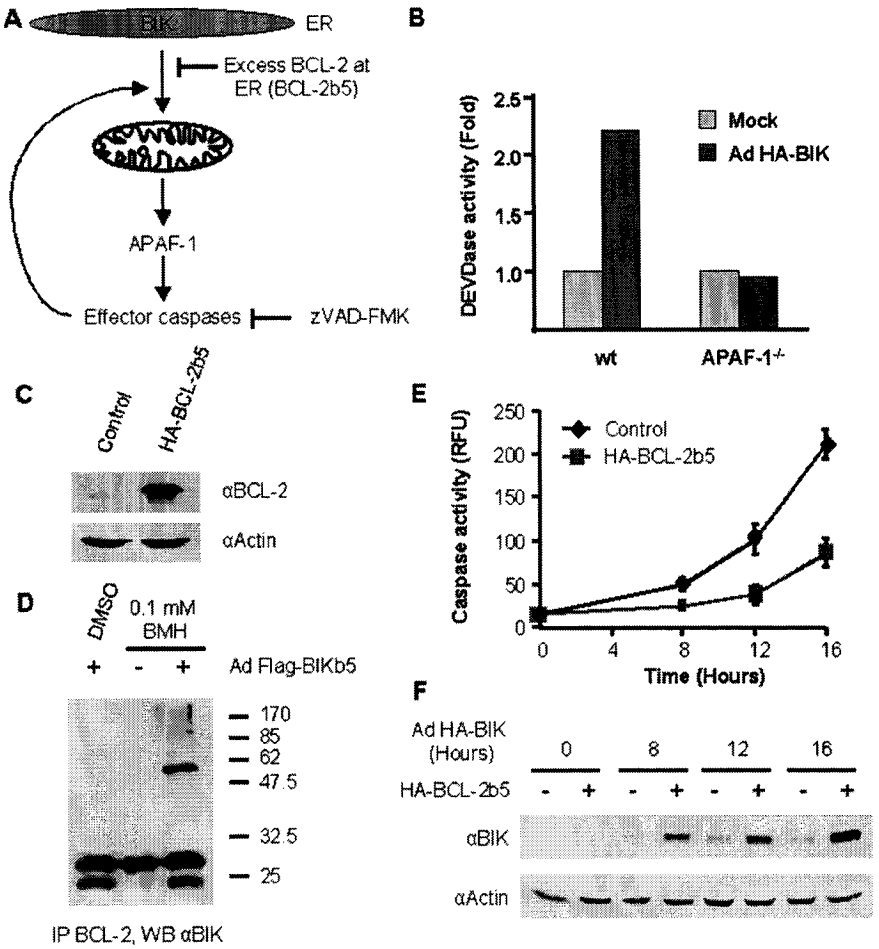


Figure 3.1 ***BIK-induced apoptosis is dependent on the APAF-1 mitochondrial pathway.*** (A) Schematic representation of the induction of apoptosis by ER-localized BIK. (B) APAF-1$^{-/-}$ cells were infected with Ad HA-BIK for 24 hours after which caspase activity was measured using DEVD-AMC. (C) Expression of HA-BCL-2b5 in stably transfected H1299 cells. 20 µg whole cell lysate were resolved by SDS-PAGE and probed with BCL-2 and actin antibodies. (D) Specific interaction between BIK and BCL-2 at the ER. H1299 cells overexpressing HA-BCL-2b5 were infected with an adenovector expressing Flag-BIKb5 for 12 hours in presence of zVAD-fmk after which the cells were fractionated. The light membrane fraction containing ER was cross-linked with 0.5 mM BMH, BCL-2 was immunoprecipited using a BCL-2-specific antibody and the immunoprecipitate was resolved by SDS-PAGE. The presence of BIK was assessed using a BIK-specific antibody. (E) Overexpression of ER-targeted BCL-2 prevents time dependant activation of caspases by Ad HA-BIK. Cells were infected with Ad HA-BIK for the indicated times after which caspase activity was measured using the fluorescent substrate DEVD-AMC. Shown are the result of three independent determinations ± S.D. (F) Expression of BIK in Ad HA-BIK treated cells. Cells were treated as in E, proteins were resolved by SDS-PAGE and probed with antibodies against BIK and actin (as a loading control)

Materials and methods

Cell culture and infection with adenoviral vectors.

H1299 lung carcinoma cells were cultured in α-minimal essential medium supplemented with 10 % heat-inactivated foetal bovine serum and 100 µg/ml streptomycin and penicillin. To generate the HA-BCL-2b5 cell lines, H1299 cells

were transfected with pcDNA 3 vector encoding HA-tagged BCL-2 mutant in which the transmembrane domain (amino acids 215-239) was replaced by the transmembrane domain of rabbit cytochrome b_5 (amino acids 107-134). G418-resistant colonies were screened for the presence of HA-BCL-2b5. Cells were infected with adenoviral vectors as described (140) using 100 plaque-forming units/cell of virus. All infections were carried in the presence of 50 μM zVAD-fmk, except for caspase activity measurements. Adenoviral vectors expressing ER-targeted Flag-BIKb5 (140), Human Noxa (114) and a mutant human Noxa in which the conserved leucine at position 29 in the BH3 domain was mutated to alanine were generated as described for wild-type BIK (59). Transfections were carried using Lipofectamine plus reagent™ (Gibco-BRL) using the previously described CFP, DRP1 and DRP1 K39E constructs (87). Caspase-3 activity essays were carried as previously described (87).

Antibodies, immunoblots and immunofluorescence

The following antibodies were used: goat anti-BIK (Santa-Cruz, CA), Hamster anti-BCL-2 (Biomol), rabbit anti BAX N-terminal epitope (Upstate), mouse anti-cytochrome c (Pharmingen), mouse anti-actin (ICN), and rabbit anti TOM20 (140). For immunoblot analysis, indicated amount of proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies. Blots were incubated with hoseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (PerkinElmer Life Sciences). For immunofluorescence, cells were grown on coverslips and treated as indicated in the figure legends. Cells were then fixed and analyzed by double label immunofluorescence using AlexaFluor 488 and 594 secondary antibodies and visualized using a Zeiss 510 confocal microscope. Images were captured and overlaid with the accompanying software. For live imaging, Coverslips were mounted in an aluminum chamber in 20 mM Hepes buffered medium (pH 7,4) and live images were taken using an Olympus IX70 inverted microscope using a Polychrome IV monochrometer and an IMAGO CCD camera from TillPhotonics (GmBH). The cells were maintained at 37 degrees and images were taken every 2 seconds for 100 or 200 frames.

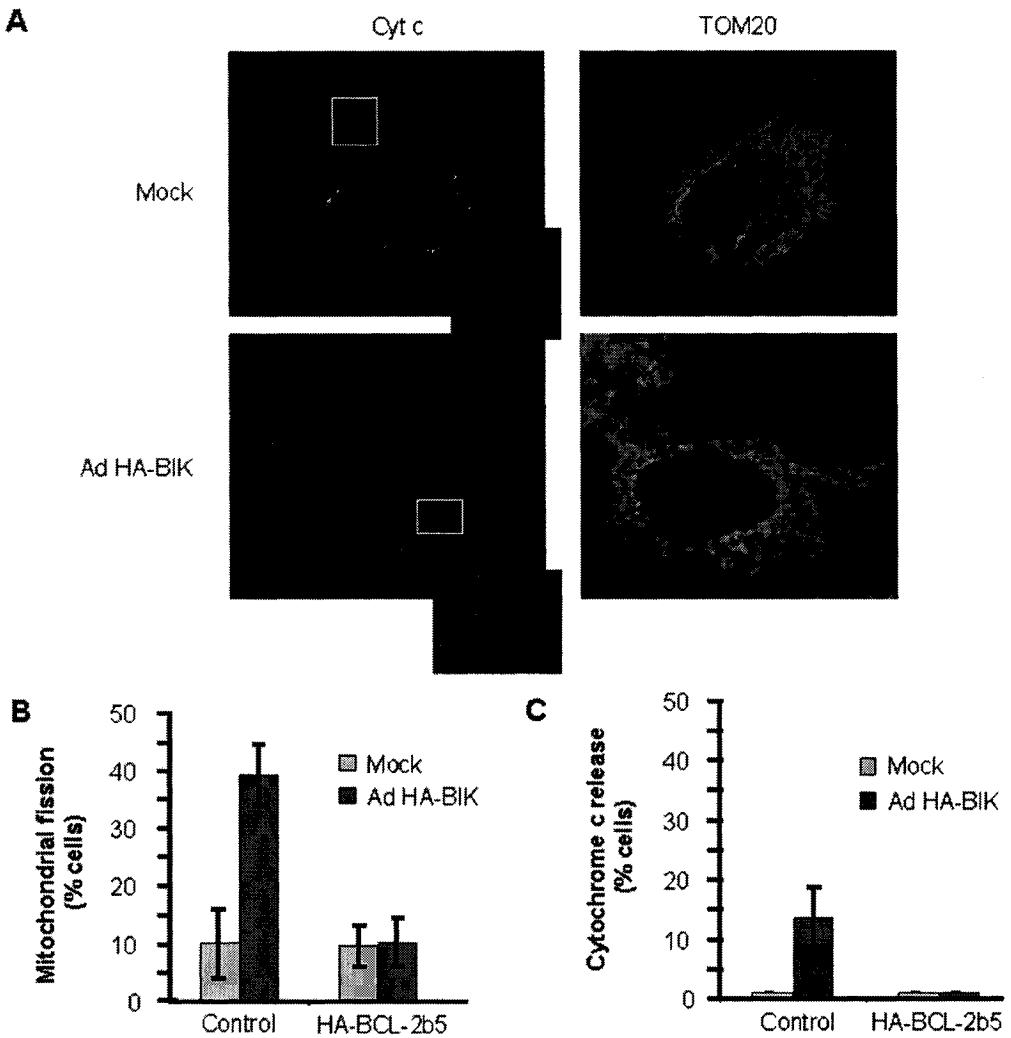


Figure 3.2 ***BIK induces changes in the morphology of mitochondria in absence of cytochrome c release.*** (A) Cytochrome c release in Ad HA-BIK-infected cells. Cells were treated with Ad HA-BIK for 12 hours in presence of zVAD-fmk, double-stained with antibodies against mitochondrial TOM20 (Alexa-Fluor 488 (Green)) and cytochrome c (Alexa-Fluor 495 (red)) and examined by immunofluorescence. Representative images are shown. (B) Quantification of mitochondrial fission from the immunofluorescence shown in A. Shown are the result of three independent determinations ± S.D. (C) Quantification of cytochrome c release from the immunofluorescence shown in A. Shown are the result of three independent determinations ± S.D.

Digitonin treatments

H1299 cells were treated with Ad HA-BIK in the presence of 50 μM zVAD-fmk for 10 hours. Mitochondria were isolated as described (140) and resuspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EGTA). Mitochondria (12.5 μg protein) in 25 μl HIM were incubated with the

indicated concentrations of digitonin for 5 min at 37°C after which mitochondria were isolated and analyzed for the presence of cytochrome c by western blot.

Electron Microscopy

Cells were trypsinized, washed in PBS, fixed in 1.6% glutaraldehyde and centrifuged at 3000 x g for 15 minutes. Cell pellets were embedded in LR white (Marivac, Québec), and thin sections were cut with a Leica Ultracut E ultramicrotome and immunolabelled with polyclonal anti GFP antibodies (Clontech) and 10nm gold-labelled secondary antibodies (Jackson laboratories). Cells were then counterstained with lead citrate and uranyl acetate to reveal those transfected with DRP1(K38E)CFP. Digital images were taken using a JEOL 1230 TEM at 60 kV adapted with a 2K x 2K bottom mount CCD digital camera (Hamamatsu, Japan) and AMT software.

Chemical cross-linking

H1299 cells overexpressing HA-BCL-2b5 were infected with Ad Flag-BIKb5 for 12 hours after which light membrane fraction (LM) was prepared as described (140) and resuspended in HIM buffer. 50 µg proteins were cross-linked with the cysteine-specific cross-linking agent *bis*-maleimiidohexane (BMH) in 100 µl HIM for 30 min after which they were centrifuged 10 min at 170,000 x g. The pellets were resuspended in 50 mM HEPES pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.5 % Triton X-100, incubated overnight with anti-BCL-2 antibody after which the antibodies were precipitated using protein G sepharose.

Ca^{2+} measurements

Measurements of ER store Ca^{2+} were carried as described previously (87, 143). Briefly, $2x10^6$ cells were resuspended in 200 µl Ca^{2+}-free buffer (20 mM HEPES pH 7.4, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 0.1 % glucose, 0.1 % BSA, 250 µM Sulfipyrazone) and loaded with 3µM Fura-2 (Molecular Probes) in the presence of 0.04 % Pluronic acid for 30 min at 37°C. Cells were then washed once and resuspended in Ca^{2+}-free buffer. $[Ca^{2+}]$ were determined with the 340 nm/380 nm exitation ratio at 510 nm emission wavelength using a LS 50B Perkin Elmer Luminescence Spectrophotometer. ER Ca^{2+} was determined as the difference in $[Ca^{2+}]$ in the absence and presence of 2 µM Thapsigargin.

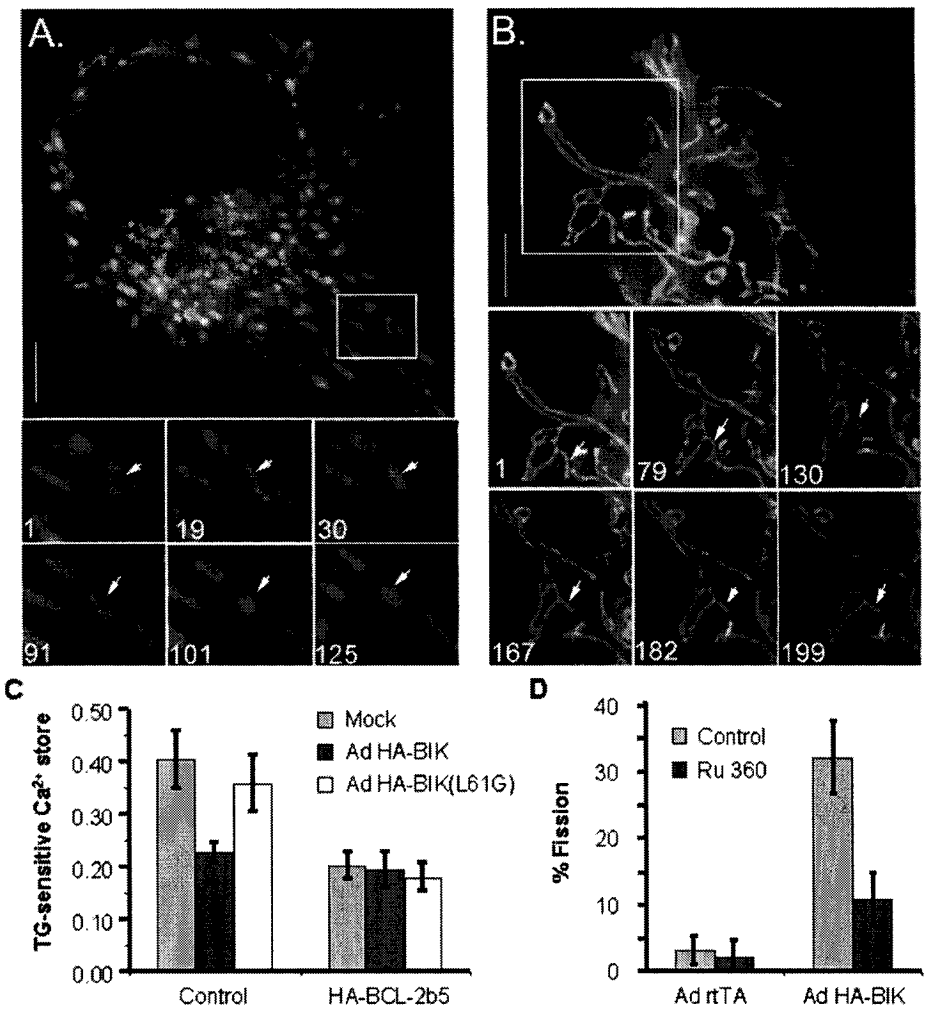


Figure 3.3 ***BIK induces DRP1-dependent mitochondrial fission that is dependent on Ca^{2+} signals.*** Cells were infected for 12 hours with Ad HA-BIK in presence of zVAD-fmk and in absence (A) or presence (B) of CFP-DRP1K38E. Mitochondria were visualized using pOCT-YFP. Bottom images are blown up from the box in the top images, the numbers indicating frame number (one frame taken each 2 sec). Representative images are shown. (C) Cells were infected for 12 hours with Ad HA-BIK in presence of zVAD-fmk, then loaded with the Ca^{2+}-sensitive dye Fura-2. Intracellular Ca^{2+} before and after addition of 2 μM tapsigargin was measured with the 340 nm/380 nm excitation ratio at 510 nm emission wavelength. Results are expressed as the difference in the 510 nm ratio before and after addition of TG. Shown are the results of at least 5 independent determinations ± S.D. (D) The mitochondrial Ca^{2+} uptake inhibitor RU360 prevents BIK-induced mitochondrial fission. H1299 cells were infected for 12 hours with either a control adenovector (Ad rtTA) or Ad HA-BIK in presence of zVAD-fmk and either the absence or the presence of 20 μM RU360. Cells were fixed, stained with the mitochondrial marker TOM20 and the percentage of cells with mitochondrial fission was determined. Shown are the result of three independent experiments.

Results

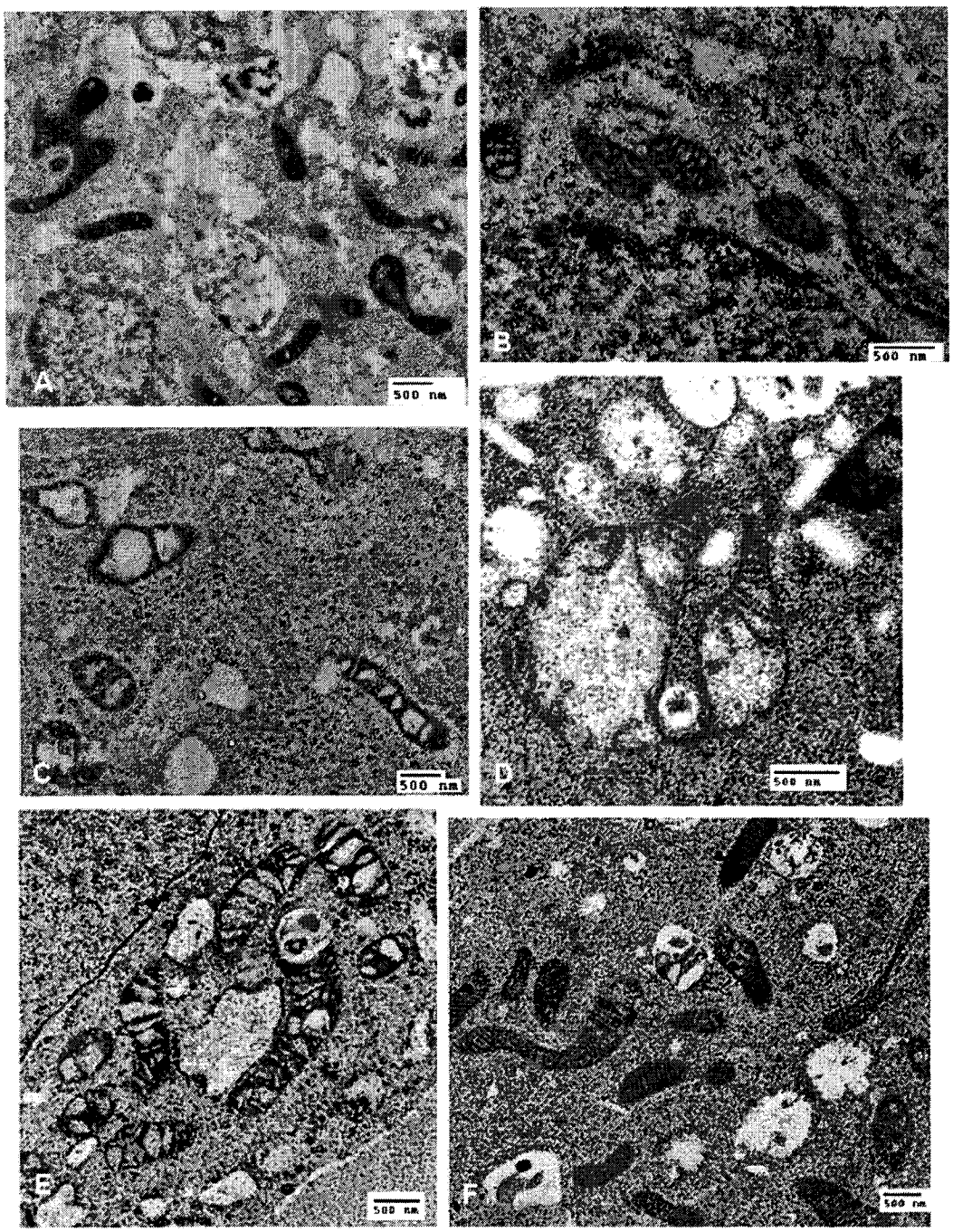
BH3-only BIK contains a hydrophobic transmembrane (TM) segment at its extreme C-terminus (120, 121). Upon induction of the endogenous protein in response to p53 stimulation or by expression from an ectopic BIK cDNA vector,

it is found primarily as an integral protein of the ER membrane (59, 140). Since BIK is a constitutively active BH3-only protein that is turned on by transcription of its gene, we created Adenovirus vectors to study the influence of this protein in isolation (59, 140). Substitution of the BIK TM with the ER-selective TM of cytochrome b_5 (Flag-BIKb5) confirmed that BIK can induce apoptosis from this ER location, independently of a direct association with mitochondria ((140) (Figure 3.1A). Importantly, however, comparisons of Wt and *Apaf-1*-null mouse embryo fibroblasts (144) infected with Ad HA-BIK indicated that this ER-initiated pathway requires downstream activation of the mitochondrial pathway for caspase activation (Figure 3.1B) and apoptosis (not shown) (Figure 3.1A,B).

Initiation of cell death by BIK can be inhibited by over-expression of BCL-2 (59, 120). To address the inhibitory effect of ER-localized BCL-2 on BIK killing, we created human H1299 (p53-null) cells stably expressing HA-BCL-2 in which the TM was replaced with that of cytochrome b5 (Figure 3.1C) and its ER location confirmed by immunofluorescence microscopy (not shown). Light membranes (LM) enriched in ER but devoid of mitochondria (140) from these cells infected with Ad Flag-BIKb5 were treated with the cysteine-specific cross-linking agent BMH in the absence of detergent, the membranes solubilized with detergent, and precipitated with antibody against BCL-2. SDS PAGE and immunoblotting of the precipitate with antibody against BIK revealed that BIK and BCL-2 at the ER membrane strongly interact (Figure 3.1D). The over-expressed HA-BCL-2b5 in these cells retarded caspase activation in response to Ad HA-BIK (Figure 3.1E). As previously documented, however, this inhibition of HA-BIK-induced caspase activity by BCL-2 also permitted a significant increase in levels of HA-BIK, which otherwise turns over during caspase-dependent cell death (Figure 3.1F, (59)).

DRP1-mediated dynamic mitochondrial transformations is an early step in response to BIK

Previous observations (59, 140) showed that, given sufficient time, BIK induces a pathway initiated from the ER that ultimately leads to mitochondria-dependent caspase activation and apoptosis. Since BIK is normally induced as part of a



cohort of p53-responsive genes, however, we sought to investigate the early steps at the ER, and therefore focused at times soon after the appearance of BIK protein in Ad HA-BIK infected cells (10-12 h, Figure 3.1F). To avoid potential influences of downstream effector caspases, resulting in possible feedback amplification of responses to BIK, all subsequent experiments were carried out in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (Figure 3.1A), which completely

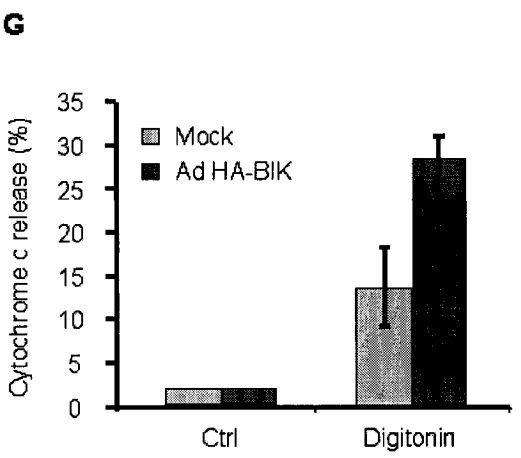


Figure 3.4 ***BIK induces DRP1-dependent remodelling of the mitochondrial cristae.*** (A)-(F) Cells were either mock infected (A-B) or infected for 10 hours with Ad HA-BIK in presence of zVAD-fmk and in the absence (C-D) or presence of CFP-DRP1-K38E (E-F), fixed and mitochondria morphology was examined by electron microscopy. DRP1-K38E positive cells were determined by immuno-gold labelling of the cells with A GFP polyclonal antibody followed by gold labelled secondary antibody (G) BIK-treated cells are more sensitive to digitonin treatments. Cells were either mock infected or infected for 10 hours with Ad HA-BIK in presence of zVAD-fmk after which mitochondria were isolated and incubated with the indicated concentrations of digitonin. The amount of cytochrome c released from the mitochondria was determined by western blotting. Shown is the average of 3 independent determinations ± S.D.

abrogated caspase-3-like (DEVDase) activity induced by BIK (data not shown; see also 140). As shown in Figure 3.2A, mitochondria that stained with an antibody against either outer membrane TOM20 or intermembrane cytochrome c were seen to undergo conversion from a tubular network to a fragmented pattern after infection with Ad HA-BIK. Quantification revealed that this fragmentation of the mitochondria occurred in about 40% of the cells by 12h infection, but less than 15% of the cells exhibited release of cytochrome c to the cytosol (Figure 3.2B,C). Of note, while numerous cells with fragmented mitochondria exhibited no cytochrome c release (Figure 3.2A), all cells that showed cytochrome c release to the cytosol also had fragmented mitochondria (data not shown), indicating that mitochondrial fragmentation preceded cytochrome c release.

Mitochondrial fission requires the activity of a dynamin related protein called DRP1 and mutation of its catalytic GTPase active site (K38E) has been shown to block mitochondrial fission and release of cytochrome c during apoptosis (86, 87). We thus used this dominant negative DRP1 mutant (DRP1-K38E) in combination with live cell imaging to assess further the nature of DRP1-mediated changes in mitochondria following infection of H1299 cells with Ad HA-BIK for 12 hours. Imaging of mitochondria in live cells was achieved by expressing a pOCT-YFP fusion protein, which is selectively targeted to the mitochondrial matrix. In response to BIK expression, the mitochondria exhibited dramatic fragmentation

(Figure 3.3A) and, of note, these fragmented mitochondria underwent multiple cycles of stretching and contraction. These dynamics are evident in the lower panels of Figure 3.3A, which capture the image of several fragments over 125 photo frames taken at 30 frames per minute. In contrast, cells infected with Ad HA-BIK in the presence of CFP-DRP1-K38E retained the mitochondrial tubular network (Figure 3.3B) typical of control cells and the dynamic mitochondrial pulsations seen with BIK alone were not observed (Figure 3.3B, bottom panel).

BIK-induced mitochondrial fission is mediated by Ca^{2+} signals

In the Fas death pathway, cleavage of BAP31 at the ER by caspase-8 induces DRP1-mediated mitochondrial fission through a pathway involving release of ER Ca^{2+} and concomitant uptake of Ca^{2+} by mitochondria (87). Expression of HA-BIK in mouse cells deleted of *Bap31* (145) caused activation of caspases (not shown) and, under the conditions employed here in human H1299 cells (i.e., inclusion of zVAD-fmk), did not result in BAP31 cleavage (140), arguing that the mitochondrial fission pathway stimulated by BIK does not involve BAP31. To examine the contribution of Ca^{2+}, however, H1299 cells were infected with Ad HA-BIK for 12 hours in the presence of zVAD-fmk, and then loaded with the Ca^{2+}-sensitive dye Fura-2 to measure cytosolic Ca^{2+} levels. ER Ca^{2+} stores were measured as the difference in $[Ca^{2+}]_{cytosolic}$ before and after the addition of thapsigargin (TG), an inhibitor of the SERCA pump that rapidly depletes ER of Ca^{2+} (87, 143). Expression of HA-BIK resulted in a reduction of ER Ca^{2+} while the BH3 mutant HA-BIK(L61G), which was expressed at higher levels than wt BIK (see (59)), had a much reduced effect (Figure 3.3C). Cells treated with the control Ad vector, Ad rtTA, were similar to mock infected cells (data not shown). Cells expressing HA-BCL-2b5, on the other hand, had lower resting TG-sensitive ER Ca^{2+} stores than control H1299 cells and these levels were not further reduced by expression of HA-BIK (Figure 3.3C). Ru360, an inhibitor of mitochondrial calcium uptake, was able to block mitochondrial fission induced by HA-BIK (Figure 3.3D). Moreover, Ru360 inhibited BIK-induced caspase (DEVDase) activation (data not shown), suggesting that the Ca^{2+} component of the BIK

pathway is important for its ability to ultimately trigger mitochondrial apoptosis in intact cells.

BIK induces DRP1-dependent remodelling of mitochondria cristae

Although DRP1-mediated transformations of mitochondria are required for cytochrome c release during apoptosis, the reason is poorly understood (86, 87, 92). It is known, however, that several apoptotic stimuli cause significant disruption of cristae junctions, resulting in expansion of the intermembrane space. As cristae are typically compact tubular structures whose narrow junctions seclude the majority cytochrome c from exposure to the intermembrane space, this increases the accessibility of cytochrome c to channels in the outer membrane (63).Given the dynamics of the mitochondrial fragments generated by BIK, however, one possibility is that these DRP1-mediated events are associated with remodelling of the inner membrane cristae.

In control H1299 cells, cristae are typically found as electron dense structure within mitochondria (Figure 3.4A), although some looser cristae can also be observed (Figure 3.4B). To examine the state of mitochondrial cristae in intact cells following infection with Ad BIK for 12 h, we employed transmission electron microscopy. BIK expression was associated with profound disruption of cristae junctions and expansion of the intermembrane space (seen as an electron transparent space in Figure 3.4C-D), suggesting that BIK signalling opens the cristae structure. Moreover, these changes induced by BIK were inhibited by CFP-DRP1-K38E. This inhibition ranged from almost complete (in which the morphology of mitochondria were indistinguishable from those in control cells, Figure 3.4F) to a partial inhibition, in which mitochondria exhibited evidence of expansion of the electron transparent intra-cristal compartment (Figure 3.4E). The variations of morphology observed within the DRP1(K38E) expressing, BIK induced cells was within the range observed in uninfected cells and likely represents different metabolic states.

To examine the possibility that BIK-induced remodelling of cristae was associated with mobilization of cytochrome c stores, mitochondria were isolated from control or BIK-stimulated cells and examined for their susceptibility to

digitonin-mediated release of cytochrome c. Digitonin is a detergent that has been widely used for controlled permeabilization of the outer membrane (OM) of isolated mitochondria (63). Titration was conducted with a fixed concentration of mitochondria protein to select a concentration of digitonin that did not cause significant release of OM TOM20, which would indicate a removal of the OM itself. In multiple independent experiments, mitochondria from unstimulated cells released about 12% of total cytochrome c, consistent with estimates that about 10-15% of the cytochrome c content of mitochondria is located in the intermembrane space (63, 91). As showed in Figure 3.4G, digitonin released more cytochrome c from BIK treated cells than control cells. The amount of cytochrome c released is consistent with about 35 % fission, assuming complete cytochrome c release in fragmented mitochondria and that 15 % of the cytochrome c was already released in cells, in line with the data presented in Figure 3.2. These results indicate that BIK expression in intact cells is in fact associated with mobilization of intra-organellar cytochrome c stores.

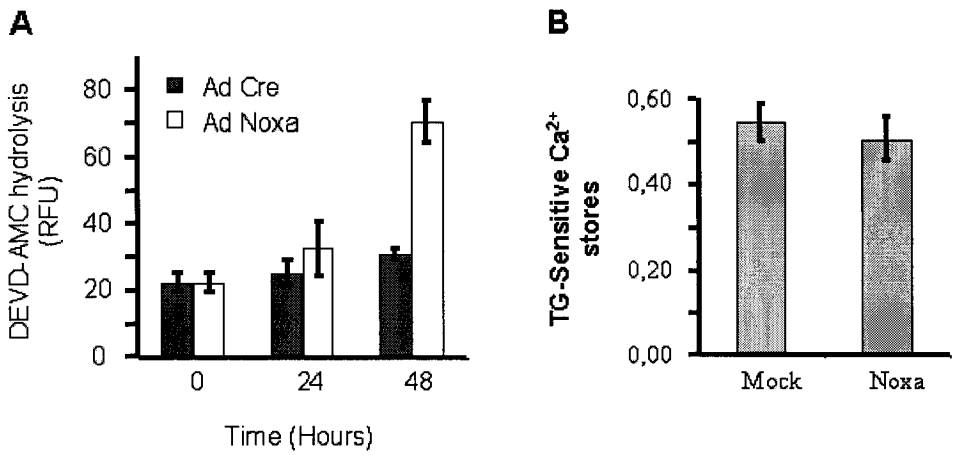


*Figure 3.5 **Noxa induces apoptosis only at late time points**.* (A) Induction of caspase activity after 48 hours infection with Ad Noxa. H1299 cells were infected with Ad Noxa or a control Ad vector (Ad Cre) for the indicated times after wich cells were collected ant analyzed for caspase-3-like activity using the fluorescent substrate DEVD-AMC. Shown is the results of 3 independent determinations ± S.D. (B) Absence of cytochrome c release by human Noxa at early time points. H1299 cells were either mock infected or infected with Ad Noxa for 12 hours in presence of zVAD-fmk after which cells were fixed and stained for cytochrome c (red) and TOM20 (green). (C) Human Noxa does not induce Ca^{2+} release from the ER on its own. ER-Ca^{2+} content was determined as in figure 3 and the results were expressed as the difference in the 510 nm ratio before and after addition of TG. Shown are the results of at least 3 independent determinations ± S.D.

BIK and NOXA cooperate to release cytochrome c from mitochondria

As Ca^{2+}-induced mitochondrial fission has previously been associated with sensitization to other mitochondrial apoptotic stimuli (87), we wanted to determine if BIK could likewise cooperate with other p53-mediators of apoptosis to induce efficient cytochrome c release and caspase activation at early time points. To this end, we chose NOXA, another BH3-only protein that is induced by p53 and has been shown to localize to mitochondria (114). Human NOXA (APR) is shorter than mouse NOXA and contains only one BH3 domain. Adenovectors expressing either the wild-type human NOXA or a mutant in which the conserved leucine at position 29 in the BH3 domain was changed to an alanine, NOXA(L29A), were generated. Infection of H1299 cells with Ad NOXA for 48 hours induced caspase activation (Figure 3.5A), but this was not observed at the early time point of 12 hours (Figure 3.4B, 3.5A). In addition, expression of human NOXA for 12 hours did not induce mitochondrial fission (data not shown) and did not affect the levels of ER Ca^{2+}stores (Figure 3.5B). In contrast, after infection of H1299 cells with both Ad HA-BIK and Ad NOXA for 10 hours, there was a significant increase in both cytochrome c release to the cytosol and caspase activation, greater that the additive effects of the two proteins expressed individually (Figure 3.6A). On the other hand, infections with twice as much of either virus alone did not result in a similar increase in caspase activation (Figure 3.6B). BIK and NOXA, therefore, can cooperate to induce efficient cytochrome c release from mitochondria. Of note, the presence of NOXA did not further increase BIK-induced ER Ca^{2+} release or mitochondrial fission (not shown). The synergy between BIK and NOXA was dependent on the presence of an intact BH3 domain in both proteins as either of the BH3 mutants, BIK(L61G) or NOXA(L29A), showed impaired cytochrome c release and caspase activation when co-expressed with its Wt partner (Figure 3.6A). Of note, no synergy was observed when NOXA was expressed with tBID (Figure 3.6C), a BH3-only protein that can promote both mobilization of intra-organelle stores of cytochrome c and its release from the organelle (46, 63).

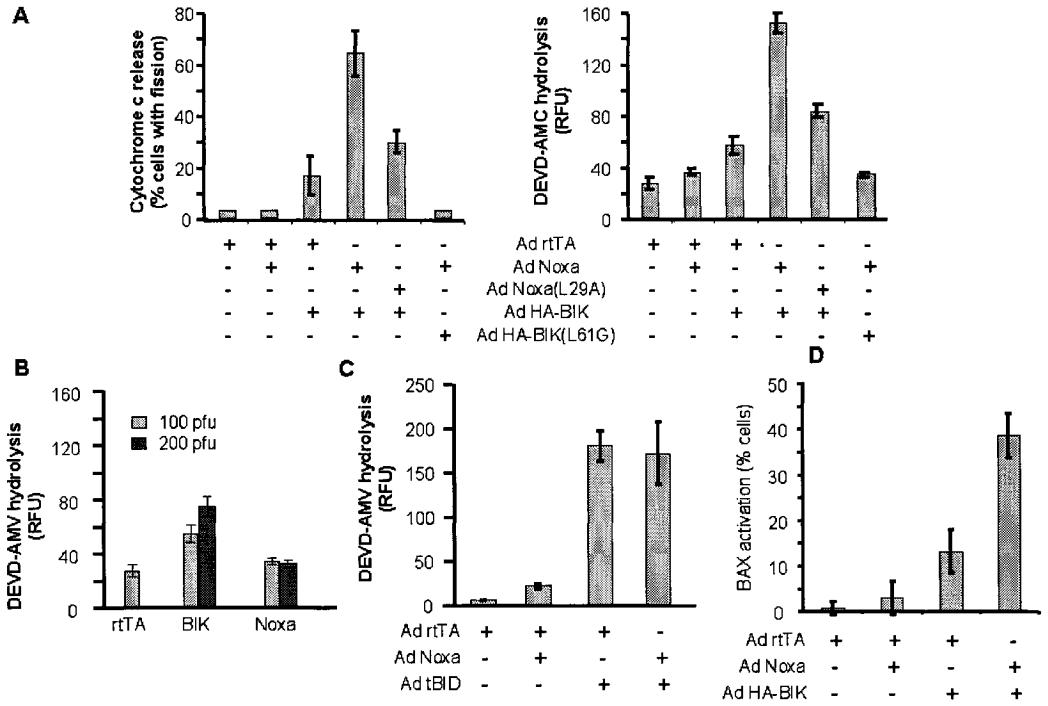


Figure 3.6 ***BIK and NOXA can cooperate to induce cytochrome c release from mitochondria.*** (A) Cytochrome c release and caspase activation by BIK and Noxa. H1299 cells were infected for 10 hours with Ad HA-BIK and Ad Noxa or their respective BH3 mutants (100 pfu/cell each virus) in the presence of zVAD. Cells were then fixed and stained with antibodies against cytochrome c and TOM20. Cells that had their mitochondria fragmented were scored for cytochrome c release. Alternatively, 25 μg lysate from cells treated in absence of zVAD-fmk were used to measure caspase-3-like activity using the fluorometric substrate DEVD-AMC. Shown are the result of at least three independent experiments ± S.D. (B) H1299 cells were infected with either 100 or 200 pfu/cell and processed as in A for measuring caspase-3-like activity. (C) Cells were treated with Ad Noxa and Ad tBID and caspase-3-like activity was evaluated as in A. (D) H1299 cells were treated and fixed as in A after which they were stained with a N-terminal specific BAX antibody. Cells positive for activated BAX were scored. Shown is the result of 3 independent determinations ± S.D.

Cytochrome c release from mitochondria requires the activation of pro-apoptotic BAX and BAK at the mitochondrial surface (46, 47). As NOXA did not affect the Ca^{2+}-dependent fission pathway of BIK, we tested whether the increase in cytochrome c release in presence of NOXA correlated with the activation of BAX. BAX stimulation results in the exposure of an epitope at the N-terminus of the protein that can be detected by immunofluorescence using an epitope- specific antibody (55, 146). Cells infected with Ad HA-BIK and Ad NOXA either alone or in combination for 10 hours were fixed, stained with the N-terminal specific antibody and BAX positive cells were scored. As with cytochrome c release, BIK and NOXA had a synergistic effect on BAX activation (Figure 3.6D).

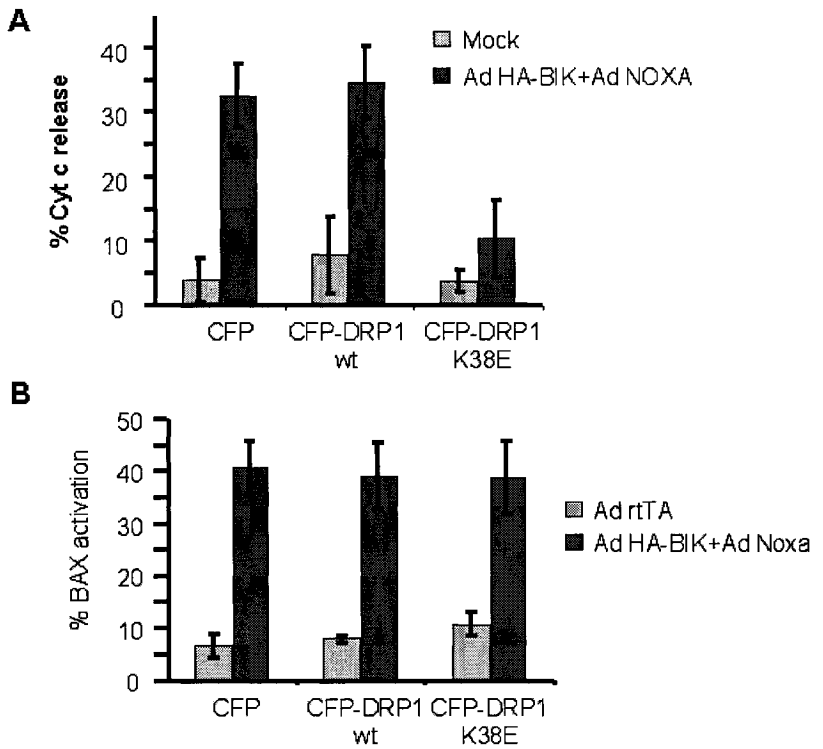


Figure 3.7 ***The cooperation between BIK and NOXA is dependent on mitochondrial fission.*** (A) DRP1 K38E prevents the induction of cytochrome c release by BIK and Noxa. H1299 cells were transfected with either CFP, CFP-DRP1 or CFP-DRP1 K38E and infected with Ad HA-BIK and Ad Noxa. Cells were fixed 10 hours post-infection and stained for cytochrome c. Result are expressed as the percent of CFP-positive cells with cytosolic cytochrome c. Shown are the results of three independent experiments ± S.D. (B) DRP1 K38E does not prevent BAX activation. Cells were treated as in B and stained with the N-Terminal specific BAX antibody and scored for the presence of activated BAX. Shown are the results of three independent experiments ± S.D.

Requirement of DRP1

Since BIK but not NOXA can stimulate mitochondrial fission, we assessed the requirement of fission for the cooperative ability of BIK and NOXA to induce cytochrome c release from mitochondria. As shown in figure 3.7A, CFP-DRP1 K38E inhibited cytochrome c release to the cytosol induced by HA-BIK and NOXA, whereas CFP and CFP-DRP1 were without effect, indicating that the synergy between these two BH3-only proteins depends on the membrane-scission competent DRP1 enzyme. In contrast, the conformational change in BAX correlating with exposure of its N-terminus was not affected by CFP-DRP1 K38E (Figure 3.7B). This is in agreement with previous results (98) and suggests that

mitochondrial fission and BAX activation are two independent events that cooperate to induce cytochrome c release from the organelle.

Discussion

Activation of p53 in response to cellular stress pathways results in the induction of multiple regulators of apoptosis (reviewed in (4)). The fact that several studies have reported that interference with a single entity among this cohort of regulators also interferes with the ability of p53 to induce apoptosis argues that several critical components may be co-operating to execute p53-mediated death in intact cells. And this appears to extend to the BH3-only family of BCL-2 regulators. For example, inhibition of p53-mediated apoptosis can be achieved by deletion of Bid in mouse embryo fibroblasts (139), by knock down of Noxa by anti-sense (114) and siRNA (118) approaches in cell lines, or by targeted deletion of Noxa (119) in mice or of PUMA in HTC116 cells (116). Similarly, knock down of p53-induced BIK in H1299 cells by RNAi resulted in a strong inhibition of p53-mediated release of Ca^{2+} from ER, egress of cytochrome c to the cytosol, and activation of caspases (Appendix 1). Thus, in the present study we employed Ad vectors to study BIK in isolation and elucidated an arm of the p53 pro-apoptotic pathway that is initiated by BIK at the ER. BIK activates an ER-mitochondrial Ca^{2+}-mediated pathway that causes DRP-1-dependent fission of mitochondria and cooperates with NOXA to elicit robust mitochondrial apoptosis.

BIK was discovered because of its interaction with cellular and viral homologues of anti-apoptoptic BCL-2 proteins (120, 121) and, as with other pro-apoptotic binding partners, its activity is strictly dependent on the ratio of BIK and BCL-2. Although much attention has been placed on the regulation of mitochondrial integrity by BCL-2 family proteins, it is now clear that this regulation also extends to the ER (8, 41, 68, 147), consistent with a critical role for the ER in supporting the mitochondrial death program. This is achieved at least in part by the influence of BCL-2 family proteins on ER Ca^{2+} stores. BCL-2 itself is particularly enriched in the ER (148) and has been shown to decrease the Ca^{2+} load of the ER, rendering cells more resistant to apoptosis (reviewed in (68)).

Excess BCL-2 at the ER of H1299 cells likewise reduced ER Ca^{2+} stores and BIK did not cause additional release to the cytosol (Figure 3.3A) correlating with a reduction in apoptosis (Figure 3.1E). Activation of pro-apoptotic BAX and BAK at mitochondria correlates with the formation of oligomeric structures with channel-like properties (50) and is required for cytochrome c release to the cytosol (46, 47). Recent evidence, however, has revealed that a small fraction of cellular BAX and BAK also resides at the ER membrane in unstimulated cells and contributes to ER calcium homeostasis (74). In contrast, when excess ER-restricted BAK (BAKb5) is delivered into doubly-deficient cells in the absence of excess BCL-2, it undergoes oligomerization and causes release of Ca^{2+} from the organelle (75). Interestingly, expression of HA-BIK did result in the recruitment and oligomerization of BAK into the ER membrane (Appendix 1), providing a possible explanation for ER Ca^{2+} release induced by BIK.

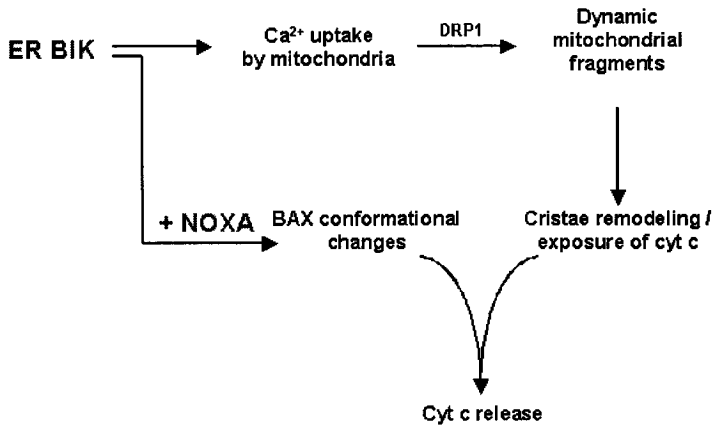


Figure 3.8 ***Model for BIK action at the ER in the context of p53 activation.***

DRP-1-mediated transformations of mitochondria are requisite for cytochrome c release to the cytosol in intact cells (86). Ca^{2+} signalling from the ER, and in particular its uptake by mitochondria, appears to provide a signal for early recruitment of DRP-1 to the mitochondrial surface during apoptosis (87, 89). Interestingly, a catalytic mutant of DRP1 inhibited mitochondrial release of cytochrome c induced by BIK and NOXA but did not interfere with the activating conformational change in BAX. DRP1-mediated fragmentation of mitochondria in response to BIK expression yields dynamic structures (Figur 3.3A) that likely

require coordinated reorganization of the inner membrane cristae (89). Opening of the cristae structure has proven necessary during apoptosis to provide a mobile pool of cytochrome c for BAX,BAK-dependent release of cytochrome c to the cytosol (63). Utilizing dominant-negative CYP-DRP1K38E, we showed here that DRP1-mediated changes in mitochondrial structure induced by BIK included cristae remodelling and that BIK expression was associated with mobilization of mitochondrial stores of cytochrome c. How DRP1, which acts at the surface of mitochondria, influences cristae remodelling, however, is not known. Cross-talk with a second dynamin GTPase, OPA1 is an attractive possibility since interference with OPA1 expression induces changes in the mitochondrial structure such as swelling and stretching (95) as well as cristae reorganization (95, 96) that are similar to what is observed during apoptosis.

In summary, our results suggest the model depicted in Fig. 8. In the pro-apoptotic mode, a BIK-regulated Ca^{2+} checkpoint induces DRP-1-mediated mitochondrial fission and cristae remodelling dependent on transmission of Ca^{2+} to mitochondria. The resulting opening of cristae mobilizes cytochrome c stores (63), and this conceivably could be aided by the dynamic stretching-contraction cycles that these fragments undergo. A second DRP1-independent event is caused by a combination of BIK and NOXA, resulting in activation of BAX, robust release of cytochrome c, and activation of caspases. This is in agreement with a recent report showing that Noxa does not kill on its own but can potentiate etoposide and UV-induced apoptosis (119). Interestingly, both these agents also induce BIK expression (Appendix 1). Altogether, these results emphasize how multiple p53-mediated pathways initiated by proteins such as the BH3-only BIK and NOXA might cooperate to induce mitochondrial apoptosis.

Acknowledgments

The authors wish to thank Peter Rippstein and Rodolfo Zunino for excellent technical help. This work was supported by operating grants from the National Cancer Institute of Canada and the Canadian Institutes of Health Research.

Chapter 4: Interactions between BH3-Only BIK and BCL-2 at the Endoplasmic Reticulum Disrupts a BCL-2 Protein Complex.

Results presented in chapters 2 and 3 demonstrate a role for BIK at the ER in inducing both cytochrome c release and Ca^{2+}-dependent mitochondrial fission. However, the mechanism through which BIK induces these events remains unclear. Since BIK was first characterized as a BCL-2-interacting protein (120, 121) and I demonstrated previously that BIK interacts directly with BCL-2 at the ER (Figure 3.1), I investigated the details of the interaction between BIK and BCL-2 at the ER as well as the functional consequences of this interaction.

Summary

Induction of apoptosis is controlled by a family of proteins homologous to the BCL-2 proto-oncogene. While *C. elegans* CED-9 prevents apoptosis by binding to the caspase adaptor CED-4, their mammalian counterparts BCL-2 and APAF-1 respectively, do not interact. Rather, mammalian BCL-2 family proteins regulate the integrity of the outer mitochondrial membrane. In addition to their mitochondrial location, BCL-2 homologues are found in the endoplasmic reticulum (ER) where they can regulate apoptosis. BIK is a BH3-only protein that, upon induction by p53, localizes to the ER where it interacts with BCL-2. In conditions where the amount of ER BCL-2 is not sufficient to prevent apoptosis induced by BIK, the latter can disrupt interactions between BCL-2 and BCL-2-associated proteins. This suggests a model for BIK activation at the ER that is reminiscent of the EGL-1/CED-9/CED-4 interactions in *C. elegans*.

Introduction

Apoptosis is an evolutionarily conserved form of programmed cell death that is primordial for development and tissue homeostasis of multicellular organisms.

Studies in the nematode *C. elegans* revealed a conserved pathway for the initiation of cell death. In this organism, developmental apoptosis is induced upon the activation of the CED-3 protease by its adaptor protein CED-4. In living cells, CED-4 is kept inactive by CED-9. Upregulation of EGL-1 in dying cells results in its binding to CED-9, freeing CED-4 and inducing apoptosis (7, 58, 64).

Components of this pathway are conserved in mammalian systems. CED-3 is part of a family of cysteine proteases, termed caspases, which execute the cell through proteolysis of a specific set of substrates (7, 58). The only known mammalian CED-4 homologue is APAF-1 which binds to and activates caspase-9 in presence of cytochrome c and dATP(7, 58). CED-9 and EGL-1 are part of the BCL-2 family of apoptotic regulators which are characterized by the presence of at least one of the four BCL-2 Homology (BH)[1] domains (40, 58). CED-9 is the *C. elegans* orthologue of mammalian anti-apoptotic BCL-2 proteins, such as BCL-2 or BCL-X_L and contains all four BH domains. BCL-2 has indeed been shown to complement CED-9 loss of function (149). EGL-1 is part of a second subgroup of BCL-2 homologues termed BH3-only proteins (40, 58). These proteins contain only the BH3 domain which is required for their pro-apoptotic activity. The BH3 domain forms an amphipathic helix that binds to a groove formed by the BH1-3 domains of anti-apoptotic BCL-2 homologues (43).

Mammalian systems contain some additional players. BAX and BAK represent a second class of pro-apoptotic BCL-2 homologues that have been found only in mammalian cells. These proteins possess BH1-3 but lack the BH4 (7, 58). Gene deletion in mice revealed that these two proteins are required for apoptosis induction through their ability to induce the release of cytochrome c release from mitochondria (45, 46). Cytochrome c release is required for formation of an active APAF-1/caspase-9 complex and apoptosis induction. Interaction between APAF-1 and cytochrome c is mediated by WD-40 repeats, absent in CED-4 (58). In addition, APAF-1 does not bind to anti-apoptotic BCL-2 homologues (7, 58). The existence of a BCL-2-regulated mammalian CED-4 protein thus remains elusive.

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[1] Abbreviations used in this paper: BH, BCL-2 Homology; BMH, *bis*-maleimidohexane; ER, Endoplasmic Reticulum; HM, Heavy Membrane fraction; LM, Light Membrane fraction

Mammalian BCL-2 homologues are thought to play a major role at the mitochondria. However, members of the three classes of BCL-2 proteins are also found at the endoplasmic reticulum (ER) where they can regulate apoptotic cell death (41, 75, 140). BIK is a good example of an ER-localized human BH3-only protein that is induced by p53 (59, 140). BIK activates apoptosis through a mitochondrial pathway that is initiated from its location at the ER (140). We show here that BIK specifically interacts with BCL-2 at the ER and that the outcome of this interaction depends on the ratio of the two proteins. High levels of BIK result in the disruption of the interactions between BCL-2 and proteins normally associated to it, leading to apoptotic cell death in a manner reminiscent of CED-9/CED-4 interactions in *C. elegans*.

Materials and methods

Cell culture and infection with adenoviral vectors.

H1299 lung carcinoma cells were cultured in α-minimal essential medium supplemented with 10 % heat-inactivated foetal bovine serum and 100 μg/ml streptomycin and penicillin. H1299 cell lines overexpressing HA-BCL-2b5 were generated as described (chapter 3). Clones that expressed low or high levels HA-BCL-2 were selected and verified for the proper ER targeting of BCL-2 by immunoflurescence. BCL-2ActA construct was kindly provided by D.W. Andrews (150) and stably transfected in H1299 cells as described for HA-BCL2b5. HA-BIK and HA-BIK(L61G) adenovirus were previously described (59, 140) Flag-BIKb5 adenoviral vector was generated as described (59) using a BIK construct in which the C-terminal transmembrane domain of BIK (amino acids 135-160) was changed for that of rabbit cytochrome b5 (amino acids 107-134) (140). Infections were carried as described (140) using 100 plaque-forming units/cell of virus in the presence of 50 μM of the general caspase inhibitor zVAD-fmk, except when caspase activity was used as a readout. Caspase-3 activity essays were performed as previously described (87).

Antibodies, immunoblots and immunofluorescence

The following antibodies were used: goat anti-BIK, rabbit anti-BCL-X and mouse anti-MCL1 (Santa-Cruz, CA); Hamster anti-BCL-2 (Pharmingen); mouse anti-HA (Babco); rabbit anti-calnexin (gift from J. Bergeron); mouse anti-actin (ICN); rabbit anti TOM20 (140). For immunoblot analysis, indicated amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with specific antibodies.

Chemical cross-linking

H1299 cells overexpressing HA-BCL-2b5 were infected with Ad Flag-BIKb5 for 12 hours after which the light membrane fraction (LM) was cross-linked using the cysteine-specific cross-linking agent *bis*-maleimidohexane (BMH) as described (chapter 3) Briefly, LM were prepared as described (140) and resuspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EGTA). 50 μg proteins were cross-linked with 0,1 mM BMH for 30 min at 25°C after which they were centrifuged 10 min at 170,000 x g. Pellets were analyzed for the presence of BIK and BCL-2 by western blot.

Results and discussion

Subcellular localization of anti-apoptotic BCL-2 homologues

Anti-apoptotic BCL-2 homologues have been reported to be localized both at the mitochondria and at the ER (35, 41). Fractionation of H1299 cells revealed that different BCL-2 proteins are distributed preferentially in specific organelles (Figure 4.1A). BCL-2 itself is highly enriched in the light membrane (LM) fraction containing ER. On the other hand, BCL-X_L and MCL-1 are present mostly in the heavy membrane (HM) fraction containing mitochondria. These results are consistent with previous results showing differences in subcellular localization of BCL-2 homologues and further suggest a specific role for each at different organelles (41).

The ER-localized BH3-only BIK was originally identified by its ability to bind anti-apoptotic BCL-2 homologues (120, 121). Overexpression studies indicated that BIK can interact with anti-apoptotic BCL-2 and BCL-X_L but not pro-

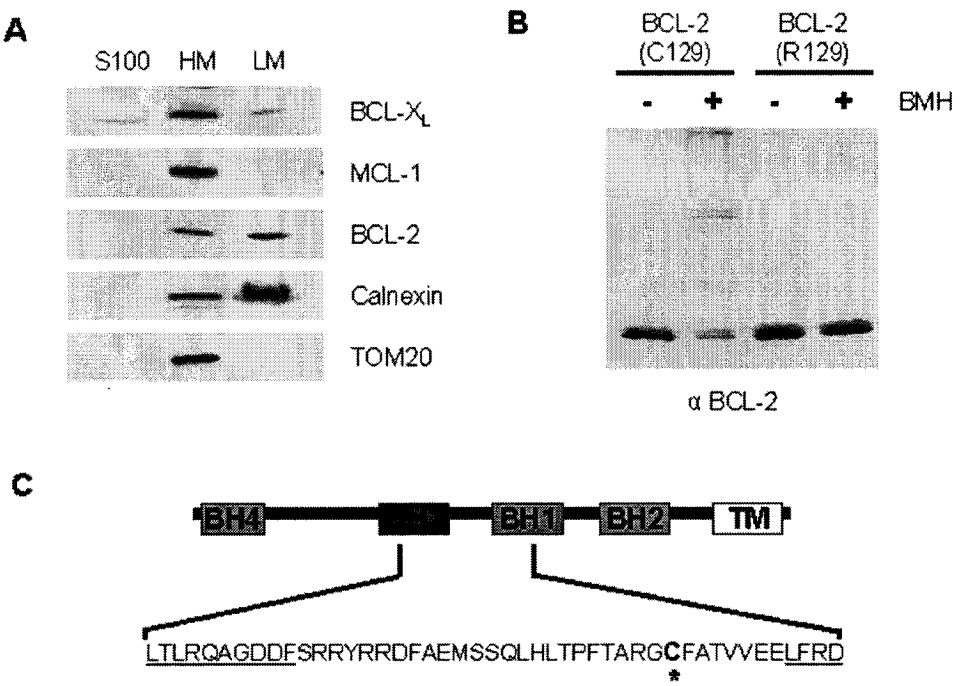

Figure 4.1 ***BCL-2 in highly enriched in the endoplasmic reticulum.*** (A) Subcellular localization of anti-apoptotic BCL-2 homologues. H1299 cells were fractionated into cytosol (S100), heavy membrane (HM) containing mitochondria and light membrane (LM) containing ER. Purity of the fractions was confirmed using antibodies against TOM20 (mitochondria) and calnexin (ER). (B) Cross-linking of BCL-2 using the cysteine-specific BMH requires the presence of cysteine 129 (BCL-2(C129)). LM fractions were prepared from H1299 cells stably transfected with either HA-BCL-2(C129) or HA-BCL-2(R129) and cross-linked using BMH. Cross-linked products were then analyzed by western blotting using an antibody against BCL-2. (C) Schematic representation of BCL-2 and the location of C129, at the edge of the BH1-2 groove. BH domains are underline in the sequence. C129 is identified by an asterisk.

apoptotic BAX (120, 121). As *in vitro* and *in vivo* experiments indicated that BIK induces apoptosis from the ER (140) and BCL-2 is the predominant anti-apoptotic BCL-2 homologue at this location in H1299 cells (Figure 4.1A), interactions between BIK and BCL-2 at the ER potentially represent an important mechanism for BIK action. In order to study more closely the interaction between BIK and BCL-2 at the ER and its functional significance, we took a cross-linking approach, using BMH as a cysteine-specific cross-linker. BCL-2 contains two cysteines, one in the transmembrane domain and the other buried in its structure. They are therefore not useful for cross-linking studies. However, reported protein sequences for BCL-2 count several polymorphic residues, among which is arginine 129 (R129). The sequence reported by the group of Stan Korsmeyer contains a cysteine at position 129 instead of the arginine (151). This cysteine is

located at the edge of the groove formed by the BH1-3 of BCL-2 and would therefore be accessible to cross-link proteins binding this groove, such as BH3-only proteins (Figure 4.1C). Of note, since BCL-2(C129) has been used extensively to block apoptosis (59, 140), this mutation is unlikely to affect its anti-apoptotic activity. While BCL-2(R129) could not be cross-linked using BMH, cross-linked products were readily detectable for BCL2(C129) (Figure 4.1B)

In order to study specifically BIK-BCL-2 interactions at the ER, we targeted BCL-2(C129) to the ER by replacing its C-terminal transmembrane domain by that of ER-localized cytochrome b5 (HA-BCL-2b5). Stable cell lines expressing different amounts of HA-BCL-2b5 were generated (Figure 4.2A) and proper intracellular localization verified by immunofluorescence (not shown). Cells expressing high amounts of HA-BCL-2b5 were resistant to Ad HA-BIK-induced caspase activation while low expressers were still sensitive (Figure 4.2B), suggesting that the ratio of BIK and BCL-2 at the ER dictates the apoptotic or survival response of the cells.

BIK disrupts the interaction between BCL-2 and its associated proteins

The light membrane fraction purified from H1299 cells is enriched in ER but does not contain mitochondria (140). Subsequent cross-linking experiments were thus conducted on LM fractions from control or Ad Flag-BIKb5-infected BCL-2b5 cells. Using this technique, we previously reported that BIK and BCL-2 specifically interact at the ER (chapter 3). We next set to study the consequences of this interaction on BCL-2.

The left panel of Figure 4.3A shows the cross-linking pattern of BCL-2 at the ER in control and BIK-treated cells. In control cells, specific cross-linked bands can be observed at molecular weights of 40 kDa, 55 kDa and 60 kDa (Figure 4.3A, asterisks). It should be noted that these cross-linked products are specific for the ER, as cross-linking of mitochondria containing BCL-2 targeted to this organelle using the transmembrane domain of ActA (BCL-2ActA) showed a different cross-linked pattern (Figure 4.3B). In addition, identical cross-linking patterns were observed using BCL-2b5 or wild-type BCL-2 isolated from LM (data not shown),

indicating that these cross-linked products are not the result of the addition of the cytochrome b_5 tail.

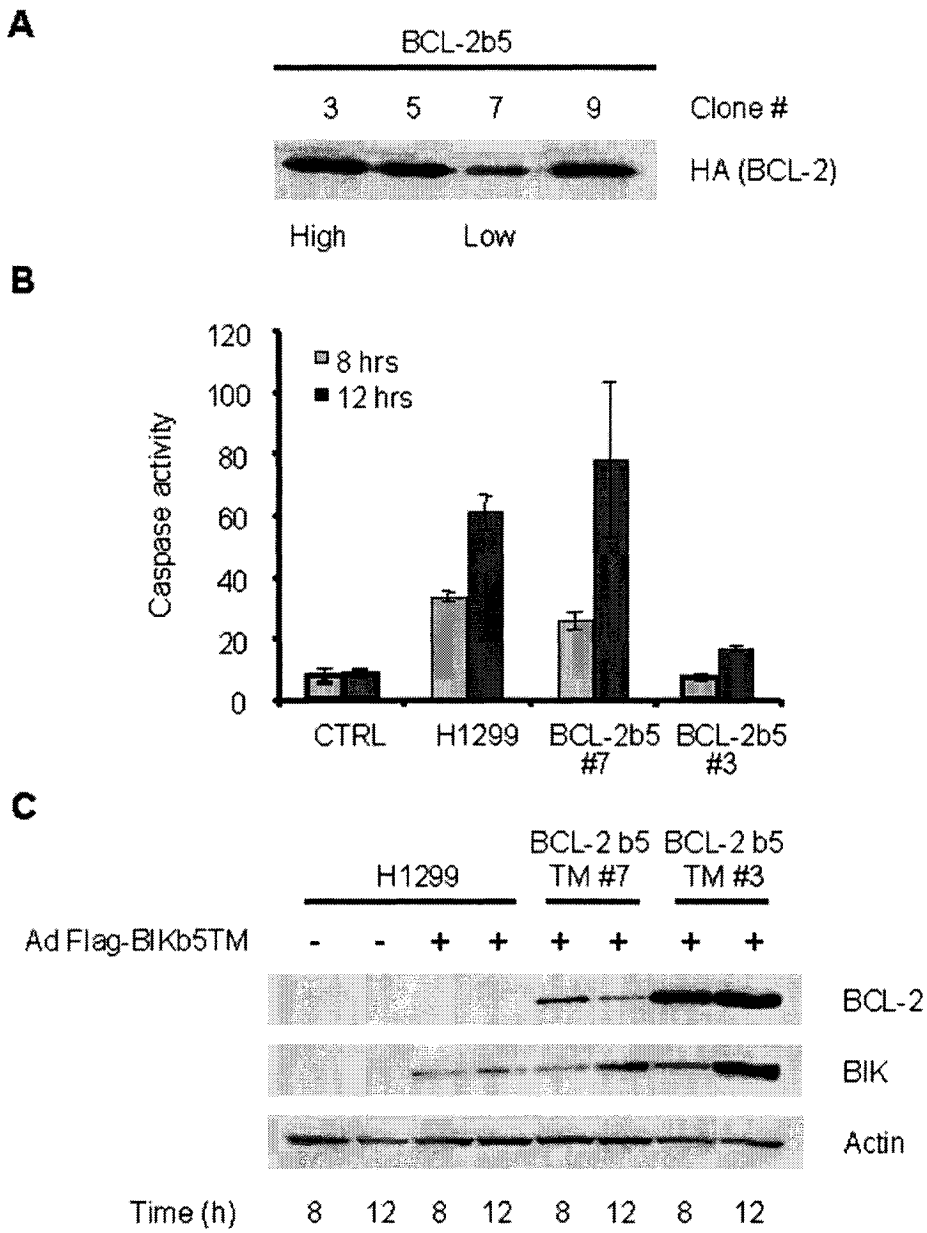

Figure 4.2 ***Induction of apoptosis by BIK depends on the ratio of BIK to BCL-2 at the endoplasmic reticulum.*** (A) Generation of H1299 cells overexpressing BCL-2 at the ER. Cells were stably transfected with HA-BCL-2b5 and tested for HA-BCL-2b5 expression using an antibody against HA. Clones expressing either low or high amounts of the transgene were selected for subsequent experiments. (B) HA-BCL-2b5 clones were infected with Ad HA-BIK for the indicated times, after which caspase-3 activity was measured using the DEVD-AMC fluorogenic substrate. (C) Cells were treated as in B, 25 μg proteins were resolved by SDS-PAGE and probed with antibodies against BCL-2, BIK and actin (as a loading control)

When HA-BCL-2b5 Low cells were infected with Ad HA-BIK, these cross-linked products disappeared and were replaced by bands co-migrating with BIK cross-linked products (Figure 4.3A, compare right and left panel), in accordance with a strong interaction between BIK and BCL-2. All experiments were carried out in presence of the general caspase inhibitor zVAD-fmk. Thus, the differences between control and BIK-infected cells are unlikely to represent caspase cleavage of the cross-linked products. BIK-induced apoptosis is dependent on its interaction with BCL-2 and both require the presence of an intact BH3 in BIK. Mutation of the conserved leucine in BIK BH3 domain (BIK(L61G)) prevents both its binding to BCL-2 and apoptosis induction. As shown in figure 4.3C, BIK(L61G) was also ineffective in displacing the BCL-2 cross-linked products. Of note, these cross-linked products are unlikely to represent BCL-2 dimers as BCL-2 does not normally form dimers (152). In addition, the size of the TAP-BCL-2 cross-linked products is inconsistent with a BCL-2 dimer (data not shown). These results indicate that direct interaction between BIK and BCL-2 disrupt the interaction between BCL-2 and proteins that are normally associated with it at the ER, interactions that likely represent important functions of BCL-2. Interestingly, the effect of BIK on BCL-2 cross-linked products was observed in BCL-2b5 low but not BCL-2b5 high clones (Figure 4.3D) and are thus dependent on the ratio of BIK to BCL-2 in a similar fashion than BIK-induced apoptosis.

Conclusions

Our study of BCL-2-interacting proteins using BMH as a cross-linker revealed that, under normal conditions, at least a portion of BCL-2 is interacting with other proteins and is thus present in a protein complex rather than being monomeric. Similar results were also obtained using two-dimension native gels (M. Nguyen, M. Germain, GC Shore; unpublished). Furthermore, the interactions reported here are specific for the fraction of BCL-2 at the ER. Formation of distinct apoptosis induction pathways, such as the caspase-2 apoptosome, have been proposed to emanate from the ER. These results thus suggest a model in which BCL-2 prevents the activation of a CED-4-like molecule. Alternatively, this released

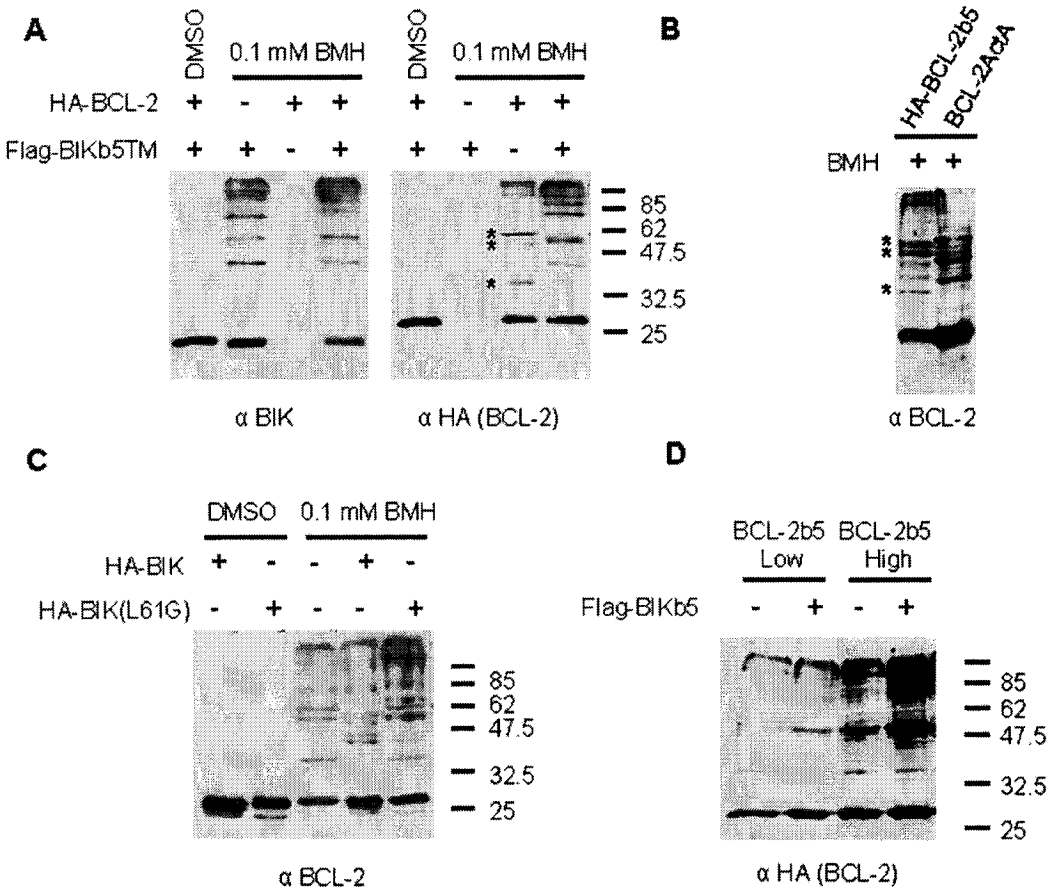

Figure 4.3 ***BIK disrupts the interaction between BCL-2 and its interacting partners.*** (A) Cells were mock- infected or infected with Ad Flag-BIKb5 for 12 hours after which LM were prepared and cross-linked using BMH. Cross-linked products were analyzed using antibodies for BIK and BCL-2. Asterisks denote the presence of the specific BCL-2 cross-linked products. (B) BCL-2-interacting proteins in the ER are different from those in the mitochondria. LM and HM fractions were prepared from HA-BCL-2b5 low and BCL-2ActA cells respectively. Each fraction was cross-linked withBMH and analyzed for the presence of BCL-2 cross-linked products using a BCL-2 specific antibody. Asterisks identify the cross-linked products as found in A. (C) Disruption of BCL-2 interactions by BIK requires an intact BH3 domain of BIK. Cells were either mock-infected or infected for 12 hours with Ad HA-BIK or Ad HA-BIK(L61G) and analyzed as in A. (D) The effect of BIK on BCL-2-interacting proteins depends on the ratio of BIK and BCL-2 at the ER. HA-BCL-2b5 low and HA-BCL-2b5 high cells were infected with Ad Flag-BIKb5 for 12 hours after which LM were prepared and analyzed as in A.

factor might be required for BAX and BAK activation or for the anti-apoptotic function of BCL-2. In either case, the results presented here suggest that one function of BIK is to disrupt the activity of BCL-2 at the ER. The identification of these proteins will thus provide useful insights into the function of BCL-2 and BIK.

Chapter 5: General Discussion

Since the discovery that cytochrome c plays an important role in the induction of apoptosis, great efforts have been made to unravel the molecular events associated with mitochondrial dysfunction during apoptosis. However, in recent years, the ER has started to attract attention, especially with the suggestion that Ca^{2+} signals are important for apoptosis progression (67, 68, 71, 74, 83, 147, 153, 154). Another element pointing to a role for the ER in apoptosis is the fact that BCL-2 family members have been found at this location (35, 41). Among these, BIK is the first BH3-only protein that has been shown to induce apoptosis from a location at the ER (Chapter 2). As BH3-only proteins are thought to ultimately be responsible for sensing cellular stresses and inducing subsequent apoptosis (40, 58, 126), this argues for a previously unrecognized fundamental role of BH3-only proteins, not only at the mitochondria, but also at the ER. That said, it should be noted that BIK is not induced by ER stress and is thus unlikely to sense ER-related events to promote apoptosis (Appendix 1). Rather, the work presented here indicates that BIK, upon induction by p53, targets the ER to activate ER-dependent apoptotic pathways that are necessary for mitochondria-dependent apoptosis. Indeed, p53-induced ER Ca^{2+} release and mitochondrial fission as well as subsequent BAX/BAK activation and cytochrome c release can all be blocked by preventing the induction of BIK using RNAi (appendix 1).

In this chapter, I will discuss ways in which BIK can influence ER-initiated apoptotic pathways and the implications in the context of p53. I would however like to begin by analyzing some characteristics of BCL-2 homologues.

BCL-2 homologues: all are not born equal

Two classes of anti-apoptotic proteins

In chapter 3, I proposed a model in which following its induction by p53, BIK-induces Ca^{2+}-dependent mitochondrial fission that sensitizes mitochondria to other pro-apoptotic insults activating BAX/BAK. This allows multiple p53-induced BH3-only proteins to cooperate in the induction of apoptosis. The

cooperation between BIK and other p53-induced BH3-only proteins was demonstrated using Noxa. Independently of the mode of action of Noxa which will be discussed below, the fact that multiple BH3-only proteins can have synergistic effects implies that all BH3 are not born equal. BH3-only proteins are cell stress sentinels that relay different stimuli to the core death machinery. The fact that each pro-apoptotic signal activates only a subset of the BH3-only proteins led to the idea that their specificity arises from their responsiveness to specific stimuli (40). I will argue that, although this is likely to play a role, a good portion of the specificity of BH3-only proteins is likely to arise from the physical properties of their BH3 domain as well as distinct subcellular localization. Some examples of preferential binding of a BH3-only protein to an anti-apoptotic protein already exist: the BH3-only BAD binds more tightly to BCL-X_L than to BCL-2 (155) while MCL-1 counters the activity of BIM but not BAD (156). The existence of activator and sensitizer BH3 domains (62) also suggests differences between BH3-only proteins.

		BH3	BH1
BCL-W	48	**AGDEFETRFRRTFSDLAAQLHVTPGSAQQRF**	TQVSDELFQ GGPNWGRLVAFFVFGAAL
BCL-X	93	**AGDEFELRYRRAFSDLTSQLHITPGTAYQSF**	EQVVNELFRDGV.NWGRIVAFFSFGGAL
BCL-2	100	**AGDDFSRRYRRDFAEMSSQLHLTPFTArgcF**	ATVVEELFRDGV.NWGRIVAFFEFGGVM
MCL-1	216	**VGDGVQRNHETAFQGMLRKLDIKNEDDVKSL**	SRVMIHVFSDGVTNWGRIVTLISFGAFV
BFL-1	40	**VAFSVQKEVEKNLKSCLDNVNVVSVDTARTLfNQVMEKEFEDGIINWGRIVTIFAFEGIL**	
		R100...D(+7)	E129...R(-2) R139...D(+6)

Table 5.1 The BH1-3 binding pocket of anti-apoptotic BCL-2 homologues and interactions with BH3 helices. Interaction data is taken from the BCL-X_L/BAK BH3 peptide structure (43). Residues in anti-apoptotic proteins are numbered according to the BCL-X_L sequence while BH3 residues are numbered according to the conserved leucine (see table 5.2). Amino acids in bold are exposed at the surface of the groove. The sequences were aligned using the program Dialign2

As mentioned in the introduction, BH3 domains are amphipathic alpha helices that bind a groove formed by the BH1, BH2 and BH3 domains of anti-apoptotic BCL-2 homologues (44). Interactions between the two molecules are mediated by hydrophobic interactions which are well conserved between anti-apoptotic BCL-2 homologues (Table 5.1; black bold residues). On the other hand, charged residues on the BH3 helix stabilize the interactions between the two proteins and are likely to be important for the specificity of the interactions. In fact, the charged residues on both the BH3 helices (Table 5.2) and at the edge of the BH1-3 binding pocket

of the anti-apoptotic proteins are poorly conserved compared to the hydrophobic residues (Table 5.1; coloured bold residues). Indeed, two groups of anti-apoptotic BCL-2 homologues can be discerned by looking at these charged residues. First, BCL-W, BCL-2 and BCL-X_L have very similar charge distribution, with R100 and E129 (BCL-X_L numbering) stabilize D(+7) and R(-2) of several BH3 helices (numbered relative to the conserved leucine, see Table 5.2). The second group is formed of MCL-1 and BFL-1, which differ from the first group for most exposed charges. Interestingly, both proteins show poor conservation of the BH4 domain despite their anti-apoptotic activity (see Figure 1.3). It is thus possible that MCL-1 and BFL-1 form a functionally distinct class of anti-apoptotic BCL-2 homologues that have their own preference for BH3-only proteins.

	-2			1			4	5	6	7	8		
BAD	Y	**G**	**R**	E	**L**	R	R	M	S	D	**E**	**F**	V	D	S
BIM	I	**A**	Q	E	**L**	R	R	I	**G**	D	**E**	**F**	N	A	Y
BMF	I	**A**	**R**	K	**L**	Q	C	I	**A**	D	Q	**F**	H	R	L
BID	I	**A**	**R**	H	**L**	A	Q	V	**G**	D	S	M	D	R	S
BIK	L	**A**	**L**	R	**L**	A	C	I	**G**	D	**E**	M	D	V	S
NOXA	C	**A**	T	Q	**L**	R	R	**F**	**G**	D	**K**	L	N	**F**	R
PUMA	I	**G**	**A**	Q	**L**	R	R	M	**A**	D	D	L	N	A	Q

Table 5.2 Sequences of BH3 domains from human BH3-only proteins. Sequences are numbered relative to the conserved leucine (L) required for BH3 function

MCL-1 has indeed been shown to act upstream of BCL-X_L during UV-induced apoptosis (157). Following UV or γ-irradiation, MCL-1 levels are rapidly decreased. This was shown to be required for subsequent translocation of BCL-X_L to the mitochondria and apoptosis induction. Further experiments suggested that MCL-1 degradation is required but not sufficient for apoptosis to occur and that downregulation of MCL-1 can sensitize cells to apoptosis.

The role of Noxa

The sensitization to apoptosis achieved by inhibition of MCL-1 is reminiscent of the pro-apoptotic activity of the p53-induced BH3-only Noxa. On its own, Noxa is a weak apoptotic inducer (Figure 3.5, (119)). However, it can cooperate with several p53-dependent pro-apoptotic insults (119) as well as p53-induced BIK (Chapter 3) to kill the cell. According to the BH3 binding specificity based on

charges described above, Noxa should bind preferentially to the second class of anti-apoptotic BCL-2 homologues. This is suggested by the fact that of all the known BH3-only proteins, Noxa is the only one with a lysine at position +7 (Table 5.1) while only BFL-1 and MCL-1 have a BH1-3 groove that can accommodate this positive charge (Table 5.1). I would thus propose that, in the context of BIK induction, Noxa acts by relieving the inhibition caused by MCL-1, allowing BAX activation, cytochrome c release and subsequent caspase activation. As MCL-1 is mostly localized at the mitochondria (Figure 4.1A), this is also consistent with the absence of effect of Noxa on ER Ca^{2+}.

A matter of location

A second point to take into consideration when studying the specificity of interactions between BCL-2 family members is their subcellular distribution. Extensive differences exist in the organelle targeted by BCL-2 homologues, this being true for all classes of BCL-2 homologues (41). For example, BCL-2 localizes mainly to the ER while BCL-X_L preferentially associates with mitochondria in H1299 cells (Figure 4.1A). Similarly, BIK localizes to the ER while most other BH3-only proteins mainly target mitochondria (40, 140). Since BIK and BCL-2 both localize to the ER at a greater extent than any other BCL-2 homologue, they are likely to be physiologically relevant binding partners, this being also supported by the cross-linking studies presented in chapter 4. As a consequence of both their binding specificities and subcellular localization, different BCL-2 homologues are likely to carry different functions, such as ER Ca^{2+} regulation, cytochrome c release or sensitization to BAX/BAK activation.

Roles of BIK at the ER

Using different approaches, I identified two distinct pathways activated by BIK at the ER: a Ca^{2+}-independent cytochrome c release activity (Chapter 2) and a Ca^{2+}-dependent mitochondrial rearrangement (Chapter 3). While the second is activated early following BIK induction and allows cooperation between BH3-only proteins, the first one likely reflects a late event that leads to BIK-induced apoptosis in the absence of other co-stimulators. Both pathways are influenced by

BCL-2 at the ER, the functional outcome being dependent on the ratio of BIK and BCL-2 at the ER surface.

The C. elegans connexion

The question thus arises of the role of this interaction in a cellular context. I would suggest that, under normal circumstances (i.e. physiological BCL-2 levels), one important role of BIK is to alter the function of BCL-2 at the ER. This is supported by several observations. First, studies in the BAX/BAK DKO mice indicated that BCL-2 acts upstream of BAX/BAK and that BCL-2 likely acts by blocking the action of these two proteins (45). Second, one of the functions of BCL-2 at the ER, Ca^{2+} regulation, is closely linked to the capacity of BCL-2 to bind BH3-only proteins. Indeed, mutations that abrogate this interaction also prevent lowering of ER Ca^{2+} stores by BCL-2 (73). In addition, BH3 domains have only been reported to interact with BCL-2 homologues and this domain is required for the killing activity of BH3-only proteins. While these observations suggest the possibility that BH3-only proteins influence the function of BCL-2, a more direct evidence came from the fact that BIK can displace other proteins from BCL-2 in a fashion that correlates with its killing activity (Chapter 4). This is very similar to the displacement of CED4 from CED9 by the BH3-only EGL1 in *C. elegans*. It should also be noted that BCL-2 has the additional function of preventing oligomerization of activated BAK, activation that is achieved by a subset of BH3-only proteins, such as tBID, that bind preferentially BAX/BAK(49). On the other hand, the overepression of BCL-2 overcomes the killing activity of BIK (Figure 4.2). This suggests that there is a threshold level that BH3-only proteins need to reach before being able to block the function of anti-apoptotic BCL-2 homologues.

In this context, one function of BCL-2 at the ER could be to sequester pro-apoptotic molecules. This is suggested by the presence of BCL-2 associated proteins that are displaced by BIK. These BCL-2-sequestered proteins could either transmit the apoptotic signal to the mitochondria, by activating BAX/BAK for example, or act at the ER. In this case, one possibility is that BCL-2 associates with components of the Ca^{2+} regulation machinery. BCL-2 has indeed been shown

to interact with SERCA2 (158), although the functional consequence of this interaction has not been defined. Such interactions would provide a mechanistic basis for the regulation of ER Ca^{2+} stores by BCL-2. Alternatively, BCL-2-interacting proteins could represent APAF-1 homologues, initiating caspase activation at the ER. The initiator caspase activated in this complex would however need to be relatively insensitive to the general caspase inhibitor zVAD-fmk as BIK-induced cytochrome c release is not blocked by zVAD-fmk (Chapter 2). Caspase-2 would thus constitute a good candidate as it is poorly inhibited by zVAD-fmk and has been shown to be required for cytochrome c release following DNA damage (159). A second possible function of BCL-2 at the ER is related to ER Ca^{2+} homeostasis. By lowering ER Ca^{2+} content, BCL-2 decreases the amount of releasable Ca^{2+}, thus blunting the apoptotic response (71). Overcoming the capacity of BIK by overexpressing BCL-2 has thus two potential effects: sequestering pro-apoptotic molecules and preventing BIK-induced release of ER Ca^{2+}. As these two functions are closely linked, it is however difficult to discern the contribution of each. This likely extents to the pro-apoptotic function of BIK, as both release of BCL-2-interacting proteins and Ca^{2+} release are dependent on an intact BH3 domain. This again suggests that the activity of BIK is dependent on the function of BCL-2 although the possibility that the BIK BH3 is required for some other function cannot be excluded at the moment.

A Ca^{2+} leak

A likely explanation for the cytochrome c releasing activity of BIK is the liberation of pro-apoptotic proteins that are normally kept inactive by interacting with BCL-2 at the ER. However, the requirement for a cytosolic factor in addition to the LM suggest that BIK relies on both factors activated at the ER and factors released into the cytosol. BIK thus does not act through a simple linear pathway. This *in vitro* work relied on isolated mitochondria which, as they are no longer forming a tubular network, are likely to be more sensitive to cytochrome c release. As a consequence, isolated mitochondria might not require massive rearrangements as needed *in vivo*. The situation is thus likely to be more complex *in vivo* as the *in vitro* experiments did not allow a study of the role of Ca^{2+}

mediated mitochondrial rearrangements caused by BIK. So how does BIK induce ER Ca^{2+} release? A first possibility is that this is achieved through the modulation of the ER Ca^{2+} regulation activity of BCL-2, as discussed above (73). A second possibility is that BIK induced ER Ca^{2+} release through activation of BAX/BAK. This arises from the observation that BAX/BAK partially localize at the ER where they can induce Ca^{2+} release (75-77). We observed that both BAX and BAK are recruited at the ER and activated following BIK induction (Appendix 1 and data not shown). It is thus possible that BIK-induced BAX/BAK activation at the ER causes Ca^{2+} release. As BIK does not bind directly to BAX/BAK, this could be achieved through the liberation of BAX/BAK-activating protein by BCL-2 upon BIK binding.

Controlling from distance: the role of Ca^{2+}

The data presented in Chapter 3 indicates that ER Ca^{2+} signals generated by BIK are taken up by the mitochondria and activate DRP1-dependent changes in mitochondrial morphology. This sensitizes cells to other pro-apoptotic stimuli. As Ca^{2+} signals are normally associated with cell growth and division (e.g. IP3 signalling)(66), the question arises of how these signals could induce both death and survival responses. The interplay between ER and mitochondria is crucial for both survival and death Ca^{2+} responses (68, 71, 83, 84) and thus, both the strength and duration of this inter-organelle communication is likely to be important. For example, thapsigargin induces a slow ER Ca^{2+} release that does not immediately induce apoptosis (71, 87). Also, in normally growing cells, IP3 signalling induces a transient ER Ca^{2+} release. Ca^{2+} is pumped back into the ER by the SERCA pump following the end of the stimulus. In contrast, induction of BIK leads to an irreversible decrease of ER Ca^{2+} content (Figure 3.3). One possible explanation for this is that binding of released cytochrome c to the IP3 receptor blocks its Ca^{2+}-dependent closure (78).

Reorganizing mitochondria: fission and cristae remodelling

Ca^{2+} released from the ER is taken up by the mitochondria where, in an apoptotic context, it induces DRP1-dependent mitochondrial fission. Using a dominant

negative form of DRP1, we and other have shown that this DRP1-dependent fission is required for cytochrome c release during apoptosis (Figure 3.7, (86, 87)). Analysis of DRP1-dependent changes in mitochondrial morphology induced by BIK Ca^{2+} signals revealed highly dynamic pulsations of the fragmented mitochondria (Figure 3.3) along with opening of the cristae structure (Figure 3.4). Interestingly, cristae remodelling has previously been shown to be blocked by the PTP inhibitor CsA (63), linking it with Ca^{2+} signals. These results thus support the hypothesis that mitochondrial fission is required during apoptosis in order to mobilize cytochrome c stores for subsequent release by BAX/BAK.

As fission is dependent on the uptake of Ca^{2+} by mitochondria (Figure 3.3, (87)), the cytosolic DRP1 is likely to be activated through a signal coming from the mitochondrial matrix rather than the cytosol. One candidate for transmitting the Ca^{2+} signals is a second dynamin related protein called OPA1. OPA1 is associated with the intermembrane side of the IMM (90, 95). Loss of the yeast homologue of OPA1, MGM1, results in fragmentation of the mitochondrial network and impaired fusion in a mating assay (Reviewed in (89, 90)), suggesting that it is a component of the fusion machinery. However, based on its submitochondrial localization and some genetic evidences, other functions have been suggested for OPA1 (reviewed in (90)). These functions include the regulation of IMM structure and coordination of fission/fusion events with the OMM (93). Indeed, downregulation of OPA1 results in early changes in the mitochondrial morphology (95) and alterations in the cristae structure similar to what is found during apoptosis (95, 96). Interestingly, overexpression of a GTPase-dead mutant of OPA1 (OPA1-K301E) resulted in enhanced cytochrome c release induced by BIK and Noxa (data not shown), suggesting that the inhibition of OPA1 might play a role in apoptosis. Thus, OPA1 is a likely candidate for relaying the Ca^{2+} signals from the matrix to cytosolic DRP1, although it is likely not the only factor involved. In addition, such a role for OPA1 in sensing Ca^{2+} signals would imply that normal mitochondrial dynamics are also influenced by Ca^{2+} signals. Indeed, IP3 signalling has been associated with sustained ATP production which is

dependent on rearrangements of the mitochondrial structure such as a 20-40% increase in mitochondria size (21).

A role for DRP1 in the function of BAX?

The electron microscopy data presented in Figure 3.4 indicates that DRP1-K38E prevents the dramatic modifications of the mitochondrial structure that occurs upon BIK induction but does not completely prevent cristae opening. In fact, partial cristae opening was also observed in control cells. This suggests that, while necessary, opening of the cristae might not be sufficient for cytochrome c release and points for a second role of DRP1 during apoptosis. As the dramatic alterations in cristae morphology induced by BIK are likely to be associated with the observed pulsations of the apoptotic mitochondrial fragments (Chapter 3), these dynamic movements could provide this second function for DRP1. It has been shown that, upon induction of apoptosis, BAX/BAK coalesces into foci that contain DRP1 and MFN2 on the mitochondrial surface, these foci being the sites for subsequent mitochondrial division (98). On the other hand, BAX/BAK can alter membrane curvature and form lipidic pores in lipid bilayers, two processes that have been linked to their ability to induce cytochrome c release (51-53). Given that BAX lipidic pores are similar to those found in membrane fusion events, it has been proposed that the fission and pore-forming activities of BAX might be linked (98). The dynamic nature of the mitochondrial fragments could thus provide a way to alter mitochondrial membrane curvature, helping BAX/BAK to release cytochrome c. It should be noted however, that while DRP1-dependent fission and BAX/BAK activation are linked mechanistically and temporally during apoptosis, they are separate processes (Figure 3.7)(87, 98).

The following model could thus be proposed. Ca^{2+} uptake by the mitochondria in a pro-apoptotic context recruits DRP1 to the mitochondrial surface through inhibition of OPA1, inducing rearrangement of both mitochondrial membranes and allowing BAX/BAK to induce cytochrome c release. Alteration in the mitochondria morphology would thus be required both for mobilization of cytochrome c and for its release. Since the PTP as well as VDAC regulate Ca^{2+} flux across both mitochondrial membranes and that BCL-2 homologues can

regulate these pores, BCL-2 family members could act on the integrity of mitochondria by both regulating Ca^{2+}-dependent fission and BAX/BAK-dependent cytochrome c release. While being in good part speculative, this model provides useful hypotheses to be tested experimentally, thus allowing a better understanding of the mechanism of mitochondrial rearrangements during apoptosis.

Under control of p53

Altogether, the results presented here indicate that the BH3-only protein BIK, upon induction by p53, targets the ER to induce apoptosis. One pathway induced by BIK through Ca^{2+} signals induces DRP1-dependent changes in the mitochondrial morphology that sensitize them to other pro-apoptotic insults. BIK can regulate this ER pathways of apoptosis induction through its association with BCL-2 at the ER. Put in the context of p53 induction, these results suggest a model in which different p53-induced genes cooperate to induce apoptosis (Chapter3).

A cohort of p53 targets

Several observations support a cooperation model rather that the induction of a super one-do-it-all protein for p53-induced apoptosis. For example, preventing the induction of most p53-induced proteins using antisense oligonucleotides, RNAi or gene deletion results in inhibition of p53-induced apoptosis (106, 114, 116, 117, 119, 129, 139). In addition, most pro-apoptotic p53 targets can be found in surviving cells undergoing cell cycle arrest, albeit not necessarily in the same cell. In fact, beside p21 which is required for p53-induced cell cycle arrest, no pro-apoptotic p53 target is consistently induced irrespective of the system studied (4). This is also in accordance with the idea that BH3 domains seem not to be all equivalent and each could potentially show different binding specificities for anti-apoptotic BCL-2 homologues. These various p53 effectors would thus act on several pathways leading to cell death. Various transcription-dependent and -independent pathways, including ER Ca^{2+} signals (Chapter 3, Appendix 1) have indeed been shown to be important for p53-induced apoptosis. Along with the fact

that p53 targets vary greatly according to the cell type and apoptotic stimulus, the cooperation between these different pathways, including those activated by BIK and Noxa, suggest a great plasticity of p53 responses which is likely to be important for its tumour suppressor activity.

A final word

In conclusion, the results presented here indicate that the ER plays an important role in the regulation of apoptotic processes. In fact, given the close physical and functional association between ER and mitochondria, this is likely to hold true for most apoptotic inducers. Known ER regulators of the apoptotic machinery are however scarce. A few candidates such as BAP31, Scotin (160) or RTNX (161) exist, but beside BAP31 which regulates Ca^{2+} signals (87) as well as activation of pro-caspase-8L (145), very little is known about their mode of action. The identification of the BH3-only protein BIK as a regulator of the ER arm of the apoptotic machinery thus provides a unique tool to study the relationship between ER and apoptosis.

Appendix 1: Human endoplasmic reticulum BIK is required for p53-induced Ca^{2+}-mediated mitochondrial fission signals and apoptosis.

I showed in Chapter 3 that BIK induces ER Ca^{2+} release which, following its uptake by mitochondria, activates mitochondrial fission. This is linked to remodeling of the mitochondrial innermembrane cristae, providing a mechanism for the observed sensitization for cytochrome c release following fission. This was exemplified in Chapter 3 by the cooperation between two p53-induced BH3-only proteins, BIK and Noxa, in the induction of cytochrome c release from mitochondria and subsequent caspase-activation and cell death. Here, RNAi was used to prevent BIK accumulation following p53 induction to study the role of BIK in the context of p53. Most of this work was carried by Jaigi Mathai and is included here for completeness. It also provide a nice validation of the work presented in Chapter 3.

Abstract

p53 mediates apoptosis in part through changes in gene expression and induction of many pro-apoptotic proteins. These include the BH3-only BCL-2 family members PUMA, NOXA, BID and human BIK. BIK is highly concentrated at the endoplasmic reticulum (ER), where it initiates release of ER Ca^{2+} stores. Despite its function at the ER, BIK expression is not induced by ER stress signals but rather responds to stimuli dependent on p53, including oncogenic (E1A) and genotoxic (radiation; doxorubicin) stress. This is in contrast to the murine ortholog, BLK, which does not respond to these stimuli. Employing siRNA knock downs, we demonstrate that p53-induced release of ER Ca^{2+} and mitochondrial apoptosis in human epithelial cells requires BIK, and that a Ca^{2+} target, DRP1, is necessary for p53-induced mitochondrial fission and release of cytochrome c to the cytosol. Recently a role for BAX and BAK at the ER has been determined

and involves regulation of ER Ca^{2+} homeostasis. Of note, p53 stimulates recruitment of BAK to the ER, and both its recruitment and assembly into higher order structures is inhibited by BIK siRNA. Human BIK, therefore, modulates ER pathways that support p53-mediated mitochondrial apoptosis.

Introduction

p53 is an important regulator of cell cycle progression and, in response to severe cell stress arising from oncogenic stimuli or genotoxic damage, p53 can also trigger cells to undergo self-elimination by apoptosis (5, 99, 100). Activation of the apoptotic program by p53 is complex and involves both transcriptional and non-transcriptional pathways (110, 162-164). Numerous p53-reponsive genes encoding pro-apoptotic proteins have been identified and in many cases these gene products, when expressed individually for sufficient periods of time, activate the apoptosis machinery. It is noteworthy that among the gene products responsive to p53 are a number of pro-apoptotic BH3-only members of the BCL-2 family, including NOXA (114, 118), PUMA (112, 113), human BIK (59, 140, 165), and BID (139). Since BH3-only proteins typically couple upstream stress stimuli to activation of the mitochondrial apoptosis pathway (166-168), these proteins likely represent important components of the apoptotic response to p53. Despite the multiple pro-apoptotic gene products that arise in response to p53, deletion of individual genes can each result in strong inhibition of p53-mediated cell death. Examples include PUMA, NOXA, and BID (115, 117, 119, 129, 139). Although one explanation might relate to the essential role of these individual proteins in cell-type or stress-type pathways activated by p53, another possibility is that p53 elicits more than one critical pro-apoptotic pathway, which must cooperate to result in a robust apoptotic outcome.

Utilizing a DNA micro-array analysis of cellular genes that are stimulated by the oncogenic E1A protein of Adenovirus, we recently identified BH3-only BIK as a strong responder in human KB epithelial cells. E1A is a potent inducer of both BIK protein and apoptosis, dependent on its ability to upregulate the levels of p53 (59). Moreover, expression of p53 in p53-null H1299 cells also induced BIK

mRNA and protein with kinetics very similar to the induction of $p21^{WAF1}$, indicating that induction of BIK expression is a rapid response to p53-mediated stress. The murine orthologue, BLK, is largely restricted to hematopoietic and endothelial cells and is not induced by genotoxic stress. Moreover gene deletion had little if any effect on the sensitivity of murine cells to genotoxic stress and animals developed normally (169). In contrast to most BH3-only proteins in mouse and man, which exhibit a high degree of amino acid sequence identity (166, 170), however, the human and mouse orthologues of BIK are only 42.5% identical, despite having very similar gene structures (171, 172). Consistent with the findings reported by Coultas et al. (169), we have found no evidence that Blk mRNA or protein is induced by either genotoxic stress or p53 expression in a variety of mouse cell lines or primary cell cultures (unpublished). Remarkably therefore, murine BLK and human BIK respond differently to stress stimuli. Consistent with the findings that human BIK may contribute to p53-mediated tumor suppression, there is reported evidence that mutation of the *BIK* gene is a frequent feature of B-cell lymphomas (173) and the chromatin locus 22q13.3, which contains Bik, exhibits deletions in human breast and colorectal cancers (174). To better understand the contribution of BIK to p53-mediated apoptosis in human epithelial cells, therefore, we utilized BIK RNA interference.

BIK contains a single transmembrane segment at its extreme C-terminus but, of note, in contrast to most BH3-only proteins, which target mitochondria, BIK is integrated almost exclusively in the membrane of the endoplasmic reticulum (ER)[1] (59, 140). Although other members of the BCL-2 family, including anti-apoptotic BCL-2 itself and the multi-domain BAX and BAK pro-apoptotic effector molecules, also target the ER (reviewed in (41)), the role of the ER in supporting efficient mitochondrial apoptosis is only now beginning to emerge (142, 175, 176). In the Fas pathway, for example, caspase-8 is activated as a

[1] Abbreviations: ER, endoplasmic reticulum; siRNA, small interfering ribonucleic acid; HA, hemagglutinin; LM, light membrane; HM, heavy membrane; rtTa, reverse tet transactivating protein; BMH, bismaleimidohexane.

proximal event (173), resulting in early cleavage and mitochondrial targeting of cytoplasmic BH3-only BID (39, 177). Simultaneously, cleavage of the resident integral protein of the ER, BAP31, takes place, generating a pro-apoptotic p20BAP31 fragment that remains anchored in the ER membrane (87, 134, 146, 178). p20 causes an early release of ER Ca^{2+} stores and concomitant uptake of Ca^{2+} by mitochondria, which triggers the recruitment of a dynamin-related GTPase, DRP1, to the organelle surface followed by mitochondrial fission (87). DRP1 is responsible for scission of the outer membrane during mitochondrial fission, a process that converts the tubular "worm-like" network of steady state mitochondria into punctiform fragments (179, 180). Mitochondrial fission is an early event in several apoptotic pathways (86) and in these pathways appears to be necessary for effective egress of cytochrome c from the organelle to the cytosol (86, 87). Cytoplasmic cytochrome c in turn becomes a critical constituent of the apoptosome, which processes and activates effector procaspases (14, 181). The contribution of DRP1-mediated events, including organelle fission, to the cytochrome c release pathway, however, remains to be determined. Intra-organellar stores of cytochrome c are normally retained within enclosed inner membrane cristae and must be first mobilized by a process involving cristae remodelling, making the protein available to be passed out of the organelle by the action of BAX or BAK (63). Several authors have suggested that DRP1 might initiate this response (87, 90, 168). If so it would provide a strong rationale for stimulating Ca^{2+} release from the ER as an early step during apoptosis, especially in cases where pathways that directly target mitochondrial apoptosis might be suboptimal. Here we demonstrate that BIK is required for such an ER pathway during p53-mediated apoptosis in human epithelial cells.

Results

BIK expression is regulated by p53 but not by ER stress.

The E1A oncoprotein stimulates p19ARF-mediated relief from turnover of p53 by MDM-2, which in turn results in the stabilization and upregulation of p53 protein

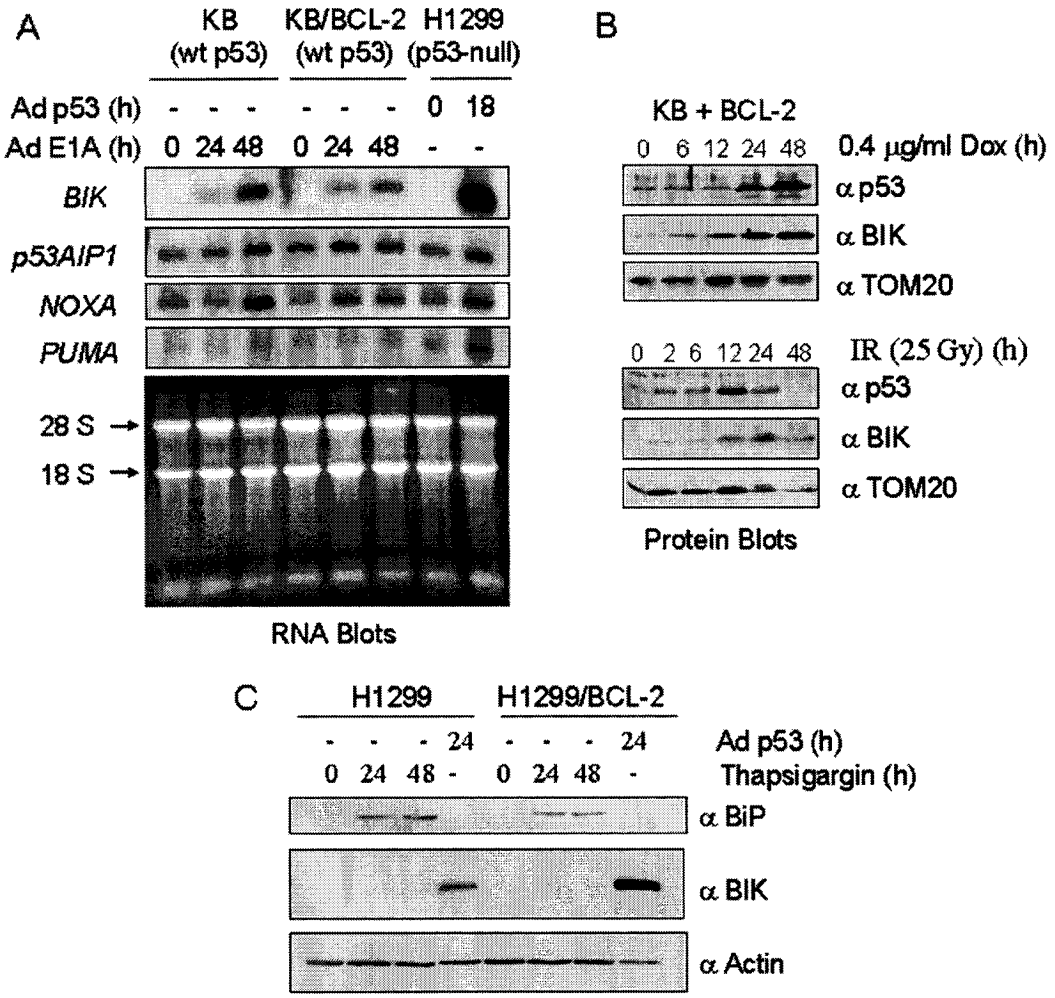

Figure ***A1 Induction of BIK mRNA and protein expression.*** A) BIK is induced by the oncogene E1A. H1299 lung carcinoma and KB oral epithelial cell lines stably expressing or not expressing HA-BCL-2 were infected for the indicated periods of time with either Ad p53 or Ad E1A vectors. Expression of the indicated genes was determined by northern blot analysis using corresponding cDNA probes (see materials and methods). The bands corresponding to 26S and 18S ribosomal RNA are indicated and provide gel loading controls. B) Activation of endogenous p53 results in BIK expression. KB cell lines expressing HA-BCL-2 were exposed to 25 Grays of gamma-radiation or treated with 0.4 µg/ml of doxorubicin. Cells were harvested at the indicated times, and BIK expression analyzed by western blot. C) ER stress does not induce BIK expression. Protein levels of BiP, BIK and actin from H1299 cells lines stably expressing HA-BCL-2, treated with either 2µM thapsigarin or infected with Ad p53 for the indicated times.

(101, 182). We have previously shown that BH3-only BIK is induced by E1A in a p53-dependent manner and that p53 is both necessary and sufficient for this induction in cultured human epithelial cells. The resulting BIK protein accumulates to high levels in cells expressing BCL-2, because BIK is induced upstream of BCL-2 and does not decay in these non-dying cells (59).

In Figure A.1A, expression of BIK mRNA in comparison to that of other p53 inducible gene products (PUMA, NOXA and p53 AIP1) was assessed by Northern blots of total RNA following infection of KB epithelial cells (p53 wt) with Ad E1A. The adenoviral vector Ad5 dl520E1B$^-$ was used for this purpose (183), which delivers only the pro-apoptotic 243 aa E1A12S oncoprotein with no E1B products, which are protective against cell death agonists. For reference, the p53 -/- human lung carcinoma cell line H1299 was infected with an adenoviral vector encoding wild-type human p53 (Ad p53). BIK mRNA was undetectable prior to delivery of E1A or p53 (time 0, Fig. A.1A). Compared to PUMA, NOXA, and p53 AIP1, the subsequent increase of *BIK* mRNA in response to these inducers, however, was robust. Since BIK protein is also induced by E1A in a p53-dependent manner (59), we also examined other stimuli that up-regulate endogenous p53 in KB cells. As shown in Figure A.1B, genotoxic damage conferred by exposure of the cells to 25 Grays of γ radiation or treatment with 0.4 μg/ml of the topoisomerase inhibitor doxorubicin also stimulated BIK protein induction in parallel with the accumulation of p53. Attempts to demonstrate similar responsiveness to such p53 stimulus by the BIK mouse orthologue BLK was without success (data not shown).

Since BIK is strongly concentrated at the ER from where it is able to exert its pro-apoptotic function independent of a mitochondrial association (59, 140), we also sought to determine whether BIK might accumulate in response to ER stress stimuli. To that end, we treated H1299 cells over-expressing BCL-2 with either Ad p53 or the ER stressor thapsigargin for the indicated times. Thapsigargin inhibits the SERCA pump, thereby preventing normal Ca^{2+} uptake into the ER from the cytosol and causing depletion of releasable ER Ca^{2+} by passive leak. Over time this leads to induction of unfolded protein response (UPR) proteins such as the chaperone BiP (Figure A.1C) and ultimately apoptosis. Even after 48 h, however, no evidence of BIK induction was observed despite the observed induction of BiP by 24 h. Similar observations were made with another ER stressor, tunicamycin, and with different cell lines. Moreover, the times at which the analyses were conducted overlapped with the appearance of dying cells (not

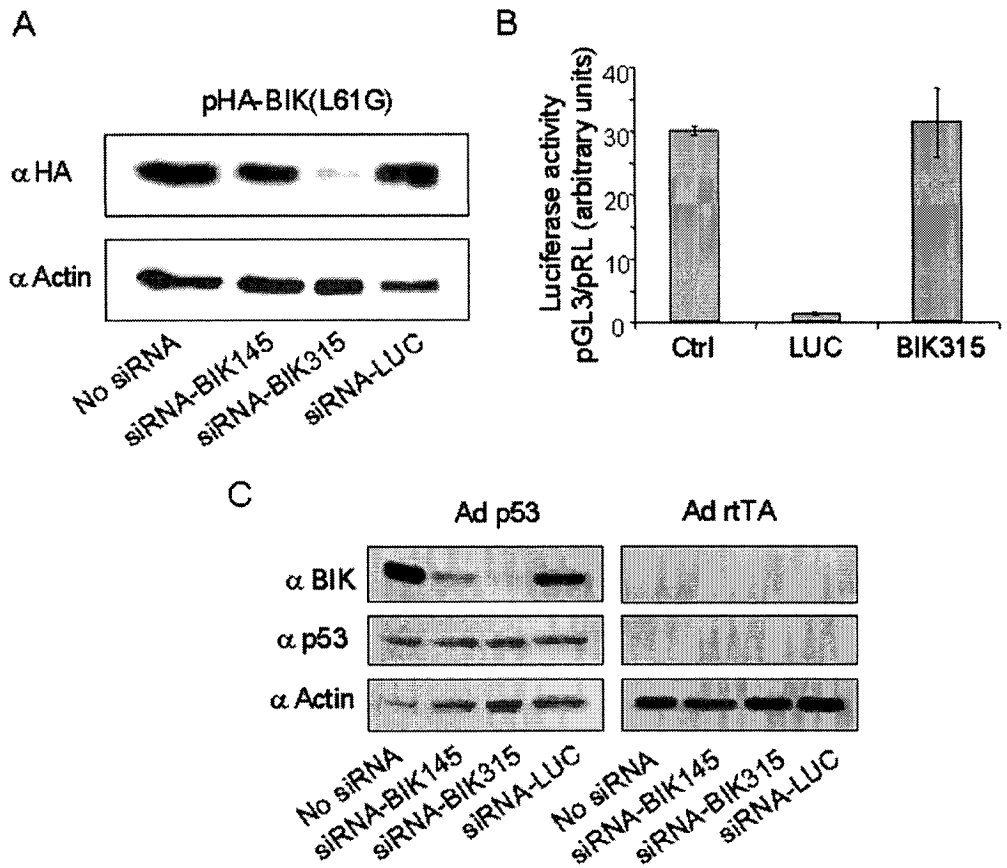

Figure A2 ***SiRNA BIK 315 specifically inhibits BIK expression.*** A) H1299 cells were transfected with an expression plasmid containing HA-BIK L61G along with siRNAs as indicated. Cells were harvested after 24 hours and total cell lysates analyzed by SDS-PAGE and immunoblotting. B) The plasmids pGL3-CMV and pRL-CMV (internal control) containing different luciferase reporter genes were co-transfected into H1299 cells along with siRNA-LUC or siRNA-BIK315. The cells were collected after 24 hours and luciferase activity measured. Shown are mean and SD of three independent experiments. C) H1299 cells were transfected with the indicated siRNAs and infected with either Ad p53 or control Ad rtTa. The cells lysates were collected and analyzed for BIK expression by SDS-PAGE and immunoblotting using the indicated antibodies.

shown). Collectively, therefore, these data indicate that BIK is up-regulated in response to a variety of classical p53 activating stimuli, but its regulation is insensitive to ER stress. This is in contrast to another p53-inducible BH3 only protein, PUMA, whose expression was shown to be induced by a number of ER stress agents in a p53-independent manner (184).

Specific Knockdown of BIK using siRNA.

To investigate the role of BIK as an effector of p53 induced apoptosis, we employed RNA interference (RNAi). To this end, we designed the small-interfering ribonucleic acid duplexes siRNA-BIK145 and siRNA-BIK315, which

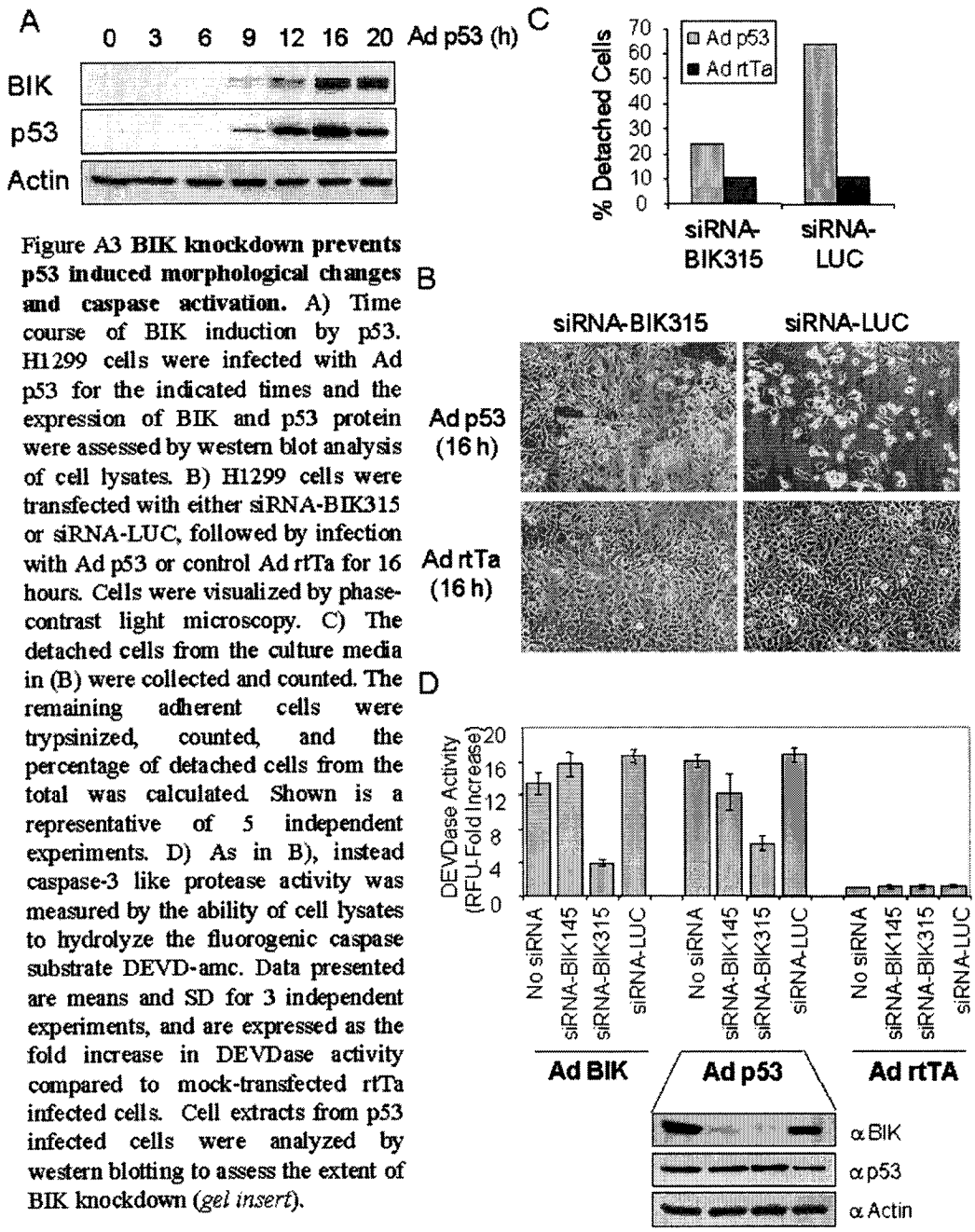

Figure A3 BIK knockdown prevents p53 induced morphological changes and caspase activation. A) Time course of BIK induction by p53. H1299 cells were infected with Ad p53 for the indicated times and the expression of BIK and p53 protein were assessed by western blot analysis of cell lysates. B) H1299 cells were transfected with either siRNA-BIK315 or siRNA-LUC, followed by infection with Ad p53 or control Ad rtTa for 16 hours. Cells were visualized by phase-contrast light microscopy. C) The detached cells from the culture media in (B) were collected and counted. The remaining adherent cells were trypsinized, counted, and the percentage of detached cells from the total was calculated. Shown is a representative of 5 independent experiments. D) As in B), instead caspase-3 like protease activity was measured by the ability of cell lysates to hydrolyze the fluorogenic caspase substrate DEVD-amc. Data presented are means and SD for 3 independent experiments, and are expressed as the fold increase in DEVDase activity compared to mock-transfected rtTa infected cells. Cell extracts from p53 infected cells were analyzed by western blotting to assess the extent of BIK knockdown (*gel insert*).

are homologous to regions within the BIK coding sequence initiating at nucleotide 145 and 315 relative to the start site of translation, respectively. An HA-BIK mutant harbouring a disabling point mutation within its BH3 region (L61G), which permits high accumulation of the protein (59), was co-transfected with siRNAs BIK315, BIK145 or a control siRNA targeting the luciferase gene within the pGL3-CMV vector (designated siRNA-LUC, Figure A.2A). SiRNA-BIK315 exhibited a strong inhibition of BIK accumulation, whereas siRNA-BIK145 was a

weaker inhibitor and siRNA-LUC showed no effect on BIK expression. The endogenous protein levels of actin were also not significantly affected by any of the siRNA duplexes. To further confirm the specificity of siRNAi-BIK315, the vector pRL-CMV, which encodes the gene for *Reneilla* luciferase, was co-transfected with the pGL3-CMV plasmid, which contains the gene for firefly luciferase. SiRNA-BIK315 or control siRNA-LUC were also included in the transfection, and the luciferase activity quantified after 24 hours. As shown in Figure A.2B, the siRNA-LUC inhibited nearly all firefly luciferase activity, whereas siRNA-BIK315 had no effect on activity as compared to the control. Thus siRNA-BIK315 is both a strong and specific inhibitor of BIK protein expression.

In Figure A.2C, the siRNAs were analyzed for their ability to knock down endogenous BIK in H1299 cells infected with Ad p53. Again, siRNA-BIK315 strongly inhibited Ad p53-induced BIK expression, whereas siRNA-BIK145 also inhibited but to a lesser extent, and siRNA-LUC had no effect. The ability of siRNA-BIK145 to inhibit induction of endogenous BIK (Figure A.2C) more effectively than its ability to counter the large amount of BIK(L61G) generated in BIK transfected cells (Figure A.2A) is consistent with siRNA-BIK145 exhibiting intermediate effectiveness against its target. Thus, siRNA-BIK315 serves as an effective means to specifically knock down expression of endogenous BIK, with siRNA-BIK145 as a potential intermediate inhibitor, and siRNA-LUC as a negative control molecule.

BIK is required for activation of caspases in response to p53.

Figure 3A shows the time course of appearance of BIK and p53 proteins following infection of p53-null H1299 cells with Ad p53; both proteins were detectable by 9 h post-infection. By 16 hours of infection with Ad p53, H1299 cells typically exhibit classical changes characteristic of the apoptotic phenotype, such as cell rounding, membrane blebbing, and activation of caspases (59) (Figure A.3B). Transfection with siRNA-BIK315 prevented these p53-induced morphological transformations from occurring at 16 h. In the presence of siRNA-BIK315, Ad p53-infected cells looked similar to those infected with adenovirus

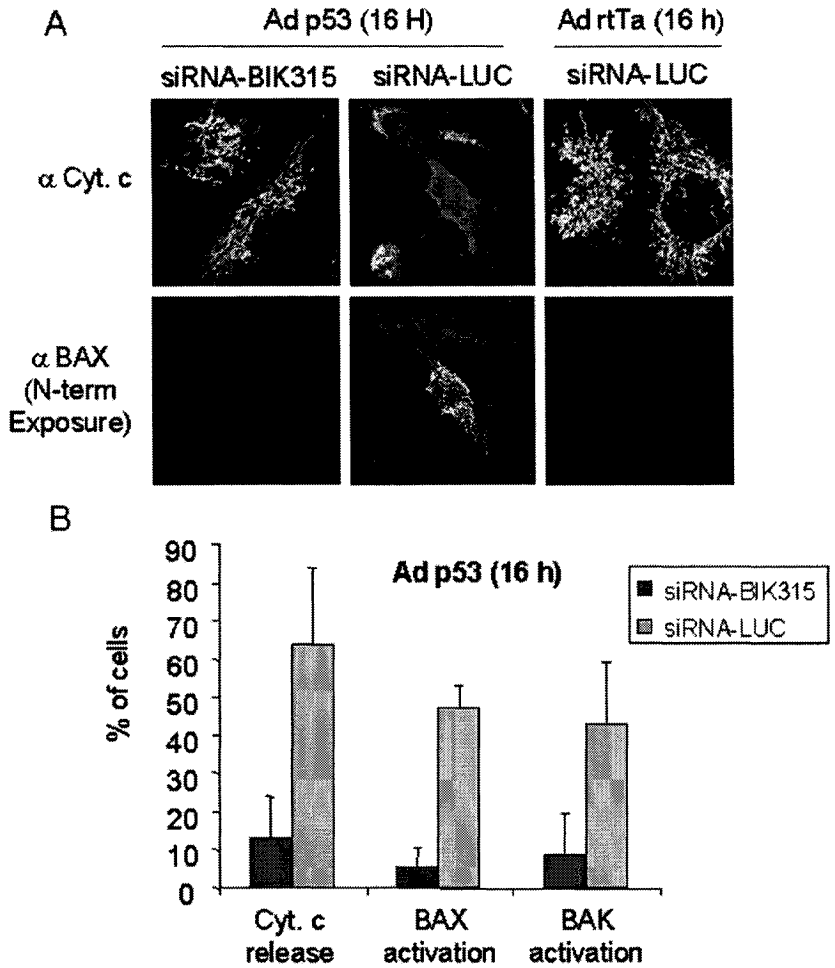

Figure A4 ***BIK knockdown decreases the amount of p53-induced cytochrome c release and BAX/BAK activation.*** A) H1299 cells were transfected with either siRNA- BIK315 or siRNA-LUC, followed by infection with Ad p53 or control Ad rtTa for 16 hours in the presence of 50uM zVAD-fmk. The cells were then fixed and stained with either the anti-cytochrome c antibody, or active conformation-specific anti-BAX or anti-BAK (not shown) antibodies. Representative images are shown. B) The cells in (A) were scored for BAX and BAK activation, as well as cytochrome c release. Shown is the mean and SD of three independent experiments.

vector encoding control reverse tet transactivating protein (Ad rtTA) (Figure A.3B), with over three times the number of cells remaining adherent to cell culture plates compared to that of the siRNA-LUC control (Figure A.3C).

Activation of effector caspases (DEVDase activity) was optimally detected by 16 h post-infection with Ad p53 (not shown). This was also attenuated by knockdown by siRNA-BIK315 of both endogenous BIK induced by Ad p53 and ectopic BIK expressed by Ad BIK (Figure A.3D). As expected, infection with control Ad rtTa vector did not result in activation of effector caspase activity. Of

note, although siRNA-BIK145 was capable of knocking down a significant fraction of the endogenous BIK that was induced by p53 (Figure A.3D, *gel insert*), substantial effector caspase activity was still observed, albeit lower than that of cells transfected with control siRNA-LUC. This is in contrast to cells in which p53-induced BIK expression was nearly completely knocked down by siRNA 3 15 (Figure A .3D, *g el i nsert*), w here the corresponding caspase activity was more strongly inhibited. Thus, there is a dose-dependent inhibition of caspase activation in response to the extent of BIK knock down, which further validates the specificity the of the BIK siRNA. Moreover, these data indicate that only small quantities of p53-induced BIK protein are sufficient to support caspase activation within the p53 death program in H1299 cells.

Since RNA interference of BIK lead to a marked decrease in effector caspase activation induced by p53 (Figure A.3D), we also sought to determine the effects of BIK knockdown on more direct mitochondrial events leading to effector caspase activation: conformational changes associated with activation of mitochondrial BAX/BAK and release of cytochrome c to the cytoplasm. H1299 cells were therefore transfected with siRNA-BIK315 and infected with Ad p53 for 16 h in the presence of 50 μM of the pan-caspase inhibitor zVAD-fmk, to minimize the effects of feedback amplification by effector caspases. (140). Quantification by immunofluorescence showed a marked decrease in the amount of p53-induced cytochrome c release to the cytoplasm compared to control A d rtTa infection or siRNA-LUC controls (Figure A.4A, *top panel*; Figure A.4B, *left*). The use of conformation specific antibodies, which recognize an exposed N-terminal epitope associated with the active forms of mitochondrial BAX (55, 146) and BAK (185) also revealed that this activation in response to p53 was mitigated by BIK knockdown because, like cytochrome c release to the cytosol, these conformational changes were strongly inhibited by siRNA-BIK315 (Fig.4A, *bottom panel*; Figure A.4B, *right*).

BIK mediates Ca^{2+} release from ER and mitochondrial fission in response to p53.

Most models of p53-mediated induction of caspase activity involve a mitochondrial pathway leading to release of cytochrome c from the organelle, an

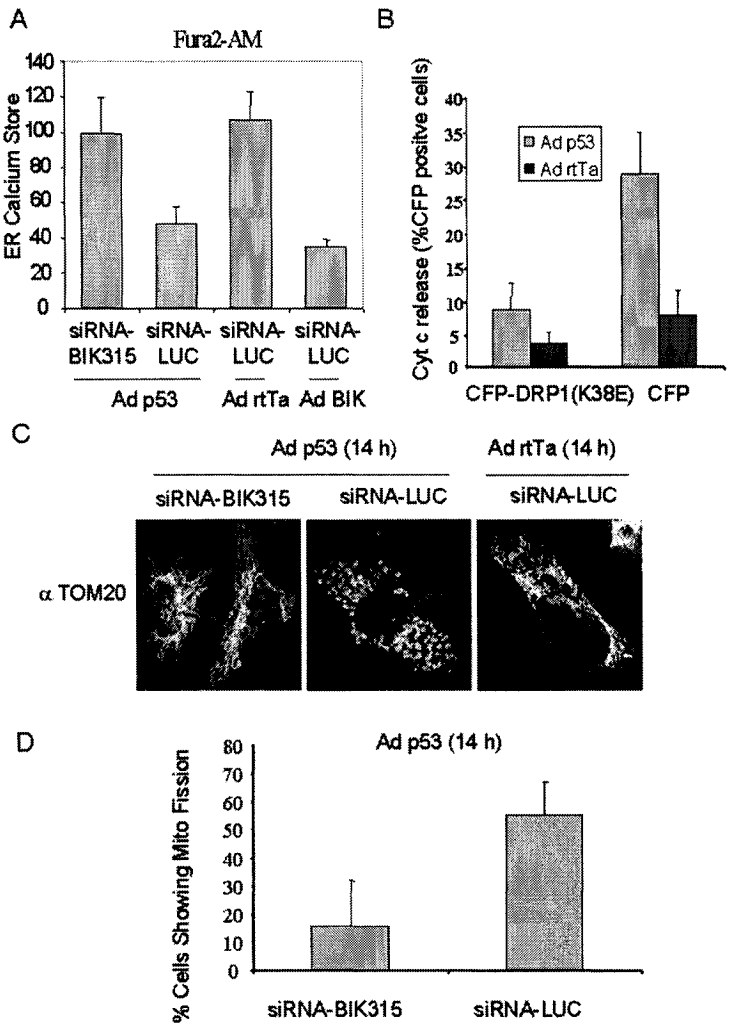

Figure A5 ***p53 regulated ER calcium sensitivity and mitochondrial fission is diminished by BIK knockdown.*** A) p53 induced ER calcium release is reduced by BIK knockdown. H1299 cells were transfected with siRNA-BIK 315 or siRNA-LUC, followed by infection with either Ad p53, Ad BIK or control Ad rtTa for 14 hours. The cells were then loaded with Fura2-AM, and ER calcium stores were measured as the difference in Fura2 fluorescence recorded after the addition of 2μM thapsigarin. Data is represented as a percentage of untreated cells. Shown are the mean and SD of 5 independent experiments. B) CFP-DRP1(K38E) prevents p53 induced cytochrome c release. H1299 cells were transiently transfected with CFP or CFP-DRP1(K38E) and subsequently infected with either Ad p53 or control Ad rtTa in the presence of zVAD-fmk. 13 hours post-infection the cells were fixed, stained with anti-cytochrome c antibody, and immunofluorescence microscopy was used to assess the distribution of cytochrome c in cells positive for CFP. Shown is the mean and SD of 3 independent experiments. C) BIK knockdown mitigates p53 induced mitochondrial fission. As in 4A, except the coverslips were fixed at 14 hours after infection with Ad p53, stained with anti-TOM 20 antibody, and the percentage of cells showing signs of mitochondrial fission was scored (D). Shown is the mean and SD of 4 independent experiments.

important cofactor that is required to activate the apoptosome (14, 99, 100). In addition, emerging evidence suggests that Ca^{2+} second messenger signalling results in mitochondrial fission following an apoptotic stimulus (87) and that mitochondrial fission is necessary for activation of the cytochrome c release pathway in diverse circumstances (86, 87). Since these ER-mediated events occur early during apoptosis and precede activation of caspases (142), we focused our analysis at 13-14 h post-infection with Ad p53.

Consistent with a role for BIK in this ER pathway, we found that infection of H1299 cells with Ad BIK induced both mitochondrial fission (not shown) as well as a robust release of Ca^{2+} from ER stores, whereas the control adenovirus vector, Ad rtTa, did not (Figure A.5A, *right*). The loss of ER Ca^{2+} was measured by loading cells with the cytosolic Ca^{2+} sensitive dye FURA-2 in the absence of

extracellular Ca^{2+}, and determining the difference in $[Ca^{2+}]_{cytosolic}$ before and after the addition of Thapsigargin, an inhibitor of the SERCA pump which causes rapid depletion of ER calcium stores (Figure A.5). Like Ad BIK, Ad p53 also induced an early loss of ER Ca^{2+} (14 h post-infection) to an extent similar to that seen for Ad BIK and, importantly, this response to Ad p53 was strongly inhibited by siRNA-BIK315 (Figure A.5A, *left*). As expected (140), the pan caspase inhibitor zVAD-fmk (50 μM) was without effect on either BIK- or p53-induced release of ER Ca^{2+}, suggesting that Ca^{2+} release from ER in response to p53 was upstream of effector caspases.

Mitochondrial fission is executed by the dynamin-related GTPase DRP1 and can be inhibited by a dominant-negative active site mutant of the enzyme, DRP1(K38E) (180, 186, 187). DRP1(K38E) also inhibits cytochrome c release from mitochondria in response to diverse stimuli (86, 87, 98). To determine if DRP1 regulated mitochondrial fission is conserved within the p53 pathway, we examined the influence of CFP-DRP1(K38E)-transfected cells at early stages of p53-induced apoptosis (13 h), compared to control CFP-transfected cells. zVAD-fmk was included to limit the influence of feedback stimulation by caspases , and the location of cytochrome c in Ad p53- or Ad rtTa-infected cells that were marked by CFP expression was determined by immunofluorescence. As shown in Figure A.5B, CFP-DRP1(K38E) inhibited the release of cytochrome c from mitochondria in response to p53. Furthermore, H1299 cells treated with siRNA-315 prior to p53 infection retained the extended mitochondrial tubular network that is typically seen in untreated cells. This is in contrast to the fragmented mitochondria seen in those cells transfected with control siRNA (Figure A.5B,C), indicating that in BIK is also required for p53-mediated fission of mitochondria.

Altogether the results indicate that BIK induction by p53 is critical for the ability of p53 to activate the mitochondrial apoptosis pathway, with Ca^{2+} and DRP1 regulated mitochondrial fission representing early steps in this process.

BIK triggers ER recruitment and oligomerization of BAK.

Although BAK is primarily a resident of the mitochondrial outer membrane, recent evidence has indicated that a fraction of BAK also resides at the ER where

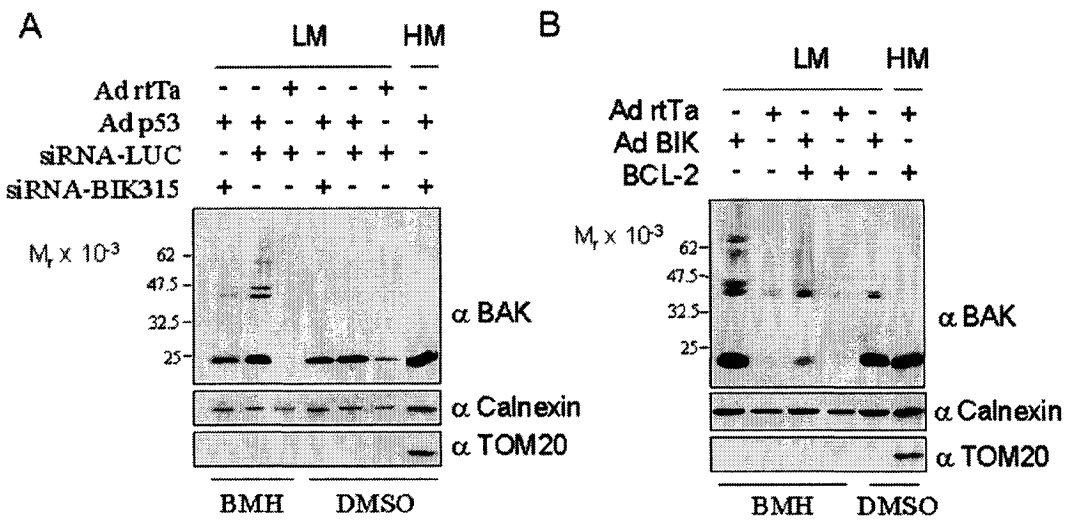

Figure A6 ***BIK promotes BAK localization and Oligomerization at the ER.*** **A) p53 induces ER BAK localization and oligomerization, diminished by siRNA-BIK315. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC, and infected with either Ad p53 or Ad rtTa in the presence of zVAD-fmk. Light membrane and mitochondrial fractions were isolated 13 hours after infection, treated with 0.5 mM BMH or DMSO, and the fractions analyzed by SDS-PAGE and probed with the indicated antibodies. B) BIK induces BAK ER localization and oligomerization, attenuated by BCL-2. H1299 cells or H1299 stably expressing BCL-2 were infected with either Ad-BIK or Ad-rtTa in the presence of 50 μM zVAD-fmk for 13 hours. ER and mitochondrial fractions were isolated and treated as in 6A).**

it can be induced to undergo oligomerization in response to stress stimuli (74-76). It has been further suggested that BAK and BAX may function at the ER to regulate its Ca^{2+} stores, thereby influencing apoptotic signals (74). Since BAK and BAX activation at mitochondria typically involves BH3-only proteins, we investigated whether BIK might induce BAK oligomerization at the ER. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC followed by infection with Ad p53 or control Ad rtTa for 13 hours in the presence of 50 μM zVAD-fmk. Light membranes (LM, enriched in ER membranes) and heavy membranes (HM, enriched in mitochondria) were collected and incubated with the sulfhydryl-reactive chemical cross-linking agent bismaleimidohexane (BMH) to cross-link oligomerized proteins. In the absence of cross-linker, we observed a strong recruitment of endogenous BAK to the LM after infection with Ad p53 (Figure A.6A, cf lanes 5 and 6). Moreover, BMH treatment of membranes resulted in the appearance of higher order BAK oligomers within this subcellular fraction following p53 expression (Figure A.6A, lane 2). The purity of the LM enrichment

is indicated by the absence of the mitochondrial outer membrane resident protein TOM 20. In Figure A.6, the exposure time of blots was selected to optimize BAK resolution; in fact, the amount of BAK that distributes to the LM following p53 stimulation is small (10 to 15%) relative to the pool that is in the HM fraction. Of note, the p53-induced BAK ER localization and oligomerization events were retarded by siRNA-BIK315 compared to control siRNA-LUC (Figure A.6A, lanes 1 and 2).

To examine the ability of BIK on its own to influence these events, control H1299 cells or H1299 cells stably expressing BCL-2 were infected for 12 hours with an adenoviral vector expressing HA-BIK or control Ad rtTa, in the presence of 50μM zVAD-fmk. Figure A.6B shows that, like p53, BIK was able to trigger BAK ER recruitment and oligomerization, events that were inhibited by the over-expression of BCL-2. Thus, BIK can replace the requirement for p53 to trigger both BAK recruitment and oligomerization at the ER.

Discussion

Although much remains to be learned about the mechanisms by which p53 induces apoptosis, a major component of its biological effect occurs through the transcriptional regulation of a number of different targets (188). Among these include the BH3-only proteins NOXA, PUMA, BID and human BIK, as well as other pro-apoptotic proteins that have no significant homology to members of the BCL-2 family, such as p53AIP1, PIDD, and PERP (59, 106, 112, 114, 129, 139, 189). Recently, a number of different co-factors and post-translational modifications of p53 have been implicated in the selectivity between its cell-cycle arrest and pro-apoptotic functions in response to DNA damage (106, 190, 191).

In the case of induction of apoptosis mediated by the activation of p53 by E1A (101, 192, 193), we found that BIK and NOXA mRNAs were stimulated by 12S E1A in KB epithelial cells whereas p53AIP1 and PUMA showed only a weak response (Fig. 1). Putative co-factors or post-translational modification of p53 could be responsible for such selection, perhaps arising from concomitant activation via other downstream targets of E1A. All of these proteins however are

known to be upregulated by p53 in response to DNA damage (114). Conversely, additional selectivity for death effectors may exist through p53-independent cell death pathways; PUMA expression, for example, has recently been shown to be responsive to ER stress, whereas BIK remains unresponsive to such stimuli (Figure A.1C).

In this study, we show that BIK is required for the full killing potential of the p53 apoptotic program in human epithelial cells. RNA interference of BIK expression resulted in strong inhibition of p53-mediated release of Ca^{2+} from ER, fission of mitochondria, changes in the conformation of mitochondrial BAX and BAK, egress of cytochrome c to the cytosol, and activation of effector (DEVDase) caspases. Emerging models emphasize the importance of ER Ca^{2+} second messenger signalling in various apoptosis pathways and this is underscored by the evidence that BCL-2 family members regulate ER calcium homeostasis (176). Over-expression of BCL-2 has been reported to lower ER calcium and diminish capacitative re-entry (72, 194), a status that is mimicked in embryonic fibroblasts with ablated BAX,BAK genes (74). Conversely, ER-targeted, oligomeric BAK results in calcium release and apoptosis (75). Analogous to the regulation of the pro-apoptotic/anti-apoptotic check point at mitochondria, BH3-only proteins may allow for BAK recruitment and oligomerization at the ER. Consistent with this model, BIK is selectively targeted to the ER (59, 140), it strongly interacts with BCL-2 at this location (Figure 3.1D, Chapter 4), and we have previously shown that indeed the ratio of BIK to BCL-2 determines cell survival or death in response to E1A (59).

Since BIK expression on its own induces early oligomerization of BAK at the ER and Ca^{2+} release from this organelle, this could contribute to p53-mediated cell killing by sensitizing the cell to other pro-apoptotic effectors that are induced by p53. This is reminiscent of the "2 hit" cooperation between ER and mitochondria in the Fas pathway, where Ca^{2+}-mediated fission of mitochondria in response to cleavage of ER BAP31 by caspase-8, together with other caspase-8-mediated events such as generation of tBID, which acts at the mitochondria (195), leads to robust release of cytochrome c to the cytoplasm (87). Selectively deleting BIK

from the p53 apoptotic program through BIK RNA interference may likewise remove an important ER pathway that supports the ability of other p53-induced pro-apoptotic effectors, such as NOXA or PUMA, to efficiently elicit mitochondrial apoptosis.

Materials and Methods

Cell Culture, plasmids and reagents.

Stable human KB oral epithelial and H1299 lung carcinoma cell lines, either expressing or not expressing ectopic HA-BCL-2 (131) were cultured in α-MEM medium supplemented with 10% fetal bovine serum and 100 mg/ml streptomycin and penicillin. Plasmids encoding CFP fused to the NH2 terminus of Drp1K38E were gifts from H. McBride (Ottawa Heart institute, Ottawa, ON). pGL3-CMV and pRL-CMV plasmids were from Promega. Carbobenzoxy-valvy-alanyl-aspartylmethyl ester-fluormethyl ketone (zVAD-fmk) was purchased from Enzyme System Products, Fura2-AM was from Calbiochem, and Doxorubicin and Thapsigargin were purchased from Sigma-Aldrich. All transfections were performed using LipofectAMINE™ Plus (Invitrogen) according to the manufacturer's protocols.

Cloning PUMA, NOXA and p53 AIP1 cDNAs and Northern blots.

PUMA, p53AIP1 and NOXA cDNA were cloned as described in (59), utilizing the sequences deposited in Genebank (accession numbers AF332558 and XM_052865, and D90070 respectively). The primers used for PUMA cloning were 5'-CGGGATCCGGTCCTCAGCCCTCGCTCTCG-3' and 5'-CGGAATTCCCGCCGCTCGTACTGTGCGTT-3'. Primers used for p53AIP1 cloning were 5'-ATGGGATCTTCCTCTGAGGCG-3' and 5'-TCACTGCAACCTCAACGGTGC-3'. Primers used for cloning of NOXA were 5'-TTGGATCCCTCCAGTTGGAGGCTGAGGTT-3' and 5'-CGGAATTCCTTGAAGGAGTCCCCTCATGC-3'. Northern blots were performed as described (59) using 30μg of total RNA extracted from H1299 cells or KB cells either expressing or not expressing ectopic HA-BCL-2.

RNAi interference of BIK and viral infection.

The following siRNA duplexes, with a 3'- end dTT overhang and corresponding to two separate regions within the BIK RNA sequence, were purchased from Dharmacon Research (Lafayette, CO) (numbers are in relation to the start site nucleotide for translation): siRNA BIK145 – AUGCAUGGAGGGCAGUGAC; siRNA BIK 315 – GUUUCAUGGACGGUUUCAC. Double stranded siRNA duplex CUUACGCUGAGUACUUCGA with a 3'end dTT overhang corresponding to a region within the luciferase gene of the pGL3 plasmid (designated siRNA-LUC) was also purchased for use as a control. The final concentration of siRNA used per transfection was 60 nM. Adenoviral infection of cells was performed approximately 12 hours after transfection with siRNA as described previously (134), using 100 plaque-forming units/cell of virus.

Antibodies, immunoblots, immunofluorescence and microscopy.

The following antibodies were utilized: goat anti-BIK (Santa-Cruz, CA), mouse anti-actin (ICN Biomedical), rabbit anti-TOM20 (described in Goping et al. 1988), monoclonal anti-p53 (PharMingen), rabbit anti-calnexin and rabbit anti-BiP (gift from J. Bergeron), mouse anti-cytochrome c (Pharmingen), rabbit anti-BAX (Santa-Cruz), and monoclonal anti-BAK (Oncogene Research Products). SDS-PAGE of whole cell lysates, transfer of proteins to nitrocellulose filters, development of blots with antibodies and detection by enhanced chemiluminescence, have been documented in earlier publications (59, 87). For immunofluoresence, cells were plated onto coverslips at approximately 50% confluency for transfection and adenoviral infection. After the indicated infection times, cells were treated and visualized as previously described (87). In experiments for Figure 5C, all cells were treated with 5μM nocodozol for 20 minutes prior to PFA fixing to aid in the visualization of fission events (186).

Luciferase Assays.

The firefly luciferase vector pGL3-CMV was transfected with Renilla luciferase vector pRL along with siRNA-LUC using Lipofectamine Plus according to the manufacturer's protocol. Cells well harvested 24 hours later and lysates assessed for luciferase activity using the Dual Luciferase Reporter Assay System

(Promega) according to the manufacturer's instruction, and a Lumat LB 9507 Luminometer.

Ca2+ measurements.

The ER calcium store was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM Thapsigargin to cells in Ca^{2+} - free buffer (87, 196). In brief, 2 X 10^6 cells were harvested and washed in Ca^{2+} - free buffer (20 mM HEPES pH 7.4, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 0.1 % glucose, 0.1 % BSA, 250 mM Sulfipyrazone). The cells were resuspended in 200 μL of calcium – free buffer containing 0.02% pluronic acid and subsequently loaded with 3mm of the cell permeable fluorescent indicator Fura2-AM for 30 minutes at 37 C. After a final wash, the cells were resuspended in Ca^{2+} - free buffer, and [Ca^{2+}] was determined with the 340 nm/380 nm excitation ratio at 510 nm emission wavelength using a LS 50B Perkin Elmer Luminescence Spectrophotometer.

Acknowledgements

We are grateful to Mai Nguyen and Imed Gallouzi for helpful discussions. J.P.M. and M.G. are recipients of the Canadian Institutes of Health Research Doctoral Research award.

This work was supported by the National Cancer Institute of Canada and the Canadian Institute of Health Research.

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