Characterization of Murine BH3-Only BLK

Samar Vikram Pai

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Department of Biochemistry
McGill University
Montreal

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ABSTRACT

BLK has 43% sequence homology to BIK and an identical BH3 domain, a region that is critical for its proapoptotic activity, making it the strongest candidate mouse ortholog. In addition, the gene structures of these two proteins are similar in functional organization. BLK and BIK activate apoptosis in similar fashion in both human and mouse cell lines, dependent on the presence of a functional BH3 domain and active caspases. BLK, like BIK, is very unstable and can be stabilized by addition of pan caspase inhibitor, zVAD-fmk. In contrast to BIK, BLK is not upregulated by p53 at the RNA or protein level in several mouse cell line systems. Furthermore, BLK localizes to the mitochondria, not to the endoplasmic reticulum where BIK resides. To further investigate the localization of BLK and BIK, the transmembrane domains of these proteins were swapped. BLKBIK and BIKBLK both localized to the endoplasmic reticulum. Enhanced Green Fluorescent Protein fused to the transmembrane domains of BLK and BIK also localized to the endoplasmic reticulum. The mitochondrial localization of BLK despite its transmembrane domain preference for the endoplasmic reticulum suggests that the N-terminal portion of BLK plays a role in targeting. Collectively, these results argue that BLK is not the functional mouse homolog of BIK.

RÉSUMÉ

La protéine de souris BLK et la proteine humaine BIK ont 43% d'homologie au niveau de la sequence acid aminé et ont un domaine BH3 identique qui est nécessaire à leur activité proapoptotique. Ceci suggère que BLK est l'orthologue de BIK dans la souris. En plus, la structure des genes de ces deux protéines ont une organisation fonctionnelle semblable. BLK et BIK induisent l'apoptose d'une manière similaire dans des lignés cellulaires humanines et murines. Dans les deux cas, le processus requière un domaine BH3 fonctionnel et des caspases actives. BLK, tout comme BIK, est une protéine tres instable qui peut être stabilisée par l'addition de l'inhibiteur pan caspases zVAD-fmk. Contrairement à BIK, le niveau d'ARN ou de protéines de BLK n'est pas augmenté par p53 dans plusieurs lignées cellulaires murines. De plus, BLK est située niveau des mitochondries, alors que BIK est situé au niveau du réticulum endoplasmique. Pour mieux élucider le méchanisme de localisation de BLK at BIK, nous avons échané leur domaine transmembranaire. Les protéines BLKBIK at BIKBLK sont toutes deux situées au niveau du réticulum endoplasmique. Les domaines transmembranaires de BLK at BIK fusionnés avec eGFP sont localisés au niveau du réticulum endoplasmique. Le fait que BLK soit localisé au niveau de la mitochondrie malgré la préférence de son doamine transmembrananire pour le réticulum endoplasmique suggère que la portion N-terminale de BLK joue un rôle dans la localisation de la protéine. L'ensemble de ces résultats suggère que BLK n'est pas l'homologue murine de BIK.

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INTRODUCTION

BACKGROUND OF APOPTOSIS

Apoptosis is a genetically controlled, biochemically executed, physiological form of cell death. Kerr and colleagues were the first to characterize this form of cell death as distinct from classical necrosis[KERR1972]. Whereas classical necrosis proceeds in an unordered manner leading to an inflammatory response, apoptosis is an organized process that occurs without inflammation.

To ensure normal development and homeostasis, the organism uses the apoptotic machinery to tightly control cell numbers and remove potentially dangerous cells. For example, the apoptotic programme is essential for proper tissue construction and maintenance[MEIER2000], as well as digit formation during embryogenesis[BAER1994]. In addition, cells that contain damaged DNA and that are beyond repair are sensed by the tumor suppressor p53, which triggers apoptosis to eliminate the threat to the organism[VOGELSTEIN2000].

Interest in apoptosis stems from its involvement in many diseases[KAM2000], since dysregulation can lead to excessive or deficient apoptosis. A mutation in any gene that is involved in coordinating the apoptotic response can lead to dysregulation. Excessive apoptosis accounts for cytopathologic effects of HIV in T cells[TERAI1991], primary neuronal cell loss and glial response in Alzheimer diseased brains[SMALE1995], and neuronal cell loss following cerebral ischemia[KRAJEWSKI1995], whereas deficient apoptosis can lead to aberrant lymphocyte accumulation in autoimmune diseases[KRAMMER2000], and tumor formation.

MORPHOLOGICAL CHARACTERISTICS OF APOPTOSIS

Apoptosis is characterized by a distinct set of biochemical and physical changes[SARASTE2000]. In contrast to classical necrosis, early events in apoptosis include loss

of cell adhesion, cell rounding, and shrinkage[KERR1994]. In the nucleus, chromatin condenses and is subsequently fragmented internucleosomally into a characteristic DNA "ladder"[WYLLIE1984] by caspase-activated Dnase (CAD)[ENARI1998] and caspase-independent endonuclease g[LI2001]. The plasma membrane undergoes blebbing in order to form apoptotic bodies that encapsulate intracellular contents. In addition, the plasma membrane exposes the aminophospholipid phosphatidylserine at its outer leaflet that triggers phagocytic removal of apoptotic bodies[VAN DEN EIJNDE1998].

APOPTOSIS IN C.ELEGANS

The nematode *Caenorhabditis elegans* can be used to study the genetic regulation of apoptosis since a complete cellular fate is available and genetic mutants are easily obtained[METZSTEIN1998]. A *C.elegans* hermaphrodite has 1090 somatic cells, 959 of which develop into defined tissues. Exactly 131 cells are programmed to undergo apoptosis in the wild type nematode. From single gene mutation analyses, the genes *egl-1*, *ced-3* and *ced-4* are required for the death of all 131 somatic cells[ELLIS1986], whereas the gene *ced-9* acts as a negative regulator of cell death. Furthermore, gene interaction studies have defined a clear apoptotic pathway in *C.elegans*[METZSTEIN1998]. In addition, the protein products of these cell-death genes interact directly. EGL-1 inhibits CED-9, CED-9 inhibits CED-4, and CED-4 activates CED-3[CONRADT1998].

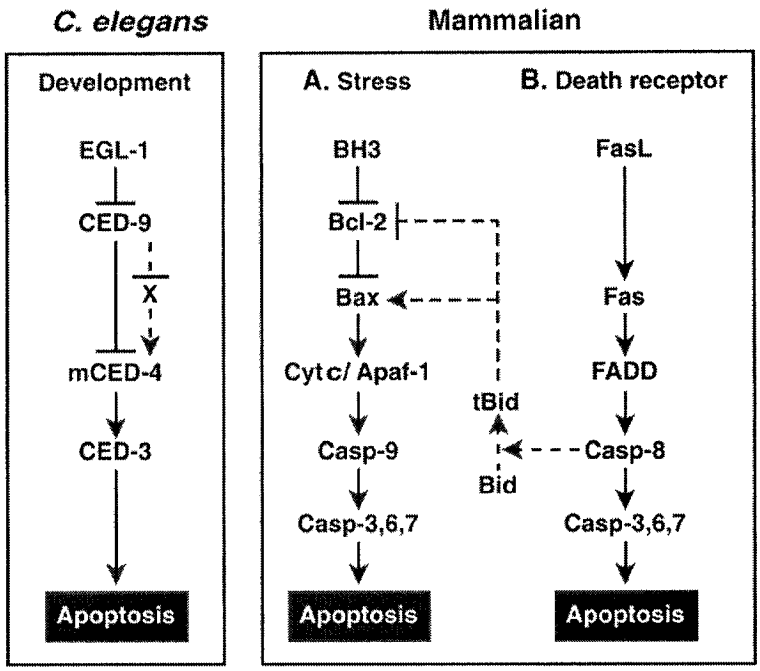

FIGURE 1.1 - *C.elegans* and mammalian apoptotic pathway. This process is evolutionarily conserved as homologs of EGL-1, CED-9, CED-4, and CED-3 have been identified in mammals (BH3-only, Bcl-2, Apaf-1, Caspase-3).(Hengartner M. 1998. *Science.* 281: 1298-1299.)

Developmental signals increase EGL-1 protein levels, which bind CED-9 in complex with CED-4, allowing CED-4 to activate CED-3 and execute apoptosis. Apoptosis appears to be evolutionarily conserved since mammalian counterparts to these proteins have been identified. EGL-1 is a member of the pro-apoptotic BH3-only subset of the Bcl-2 family of proteins; CED-9 belongs to the anti-apoptotic subset of the Bcl-2 family; CED-3 belongs to the caspase family: proteases responsible for the execution phase of apoptosis[YUAN1990]; and CED-4 is homologous to Apaf-1, an adaptor protein involved in caspase activation. Although additional players and regulation exist in humans, the basic apoptotic pathway can be studied in *C.elegans*.

CASPASES AND CASPASE SUBSTRATES

Caspases, highly conserved through evolution, are cysteine proteases that cleave substrates after aspartate residues. Substrate specificity is determined by the four residues preceding the cleavage site aspartate residue[THORNBERRY1997].

Like most proteases, caspases are synthesized as inactive zymogens known as procaspases. Procaspases are composed of three distinct functional modules: N-terminal prodomain, a large 20 kDa subunit (p20), and a small 10 kDa subunit(p10)[WOLF1999]. Caspase cleavage sites between these domains permit formation of the mature, active form of the enzyme, which is a heterotetramer containing two p20/p10 heterodimers and two active sites[EARNSHAW1999].

There are three classes of caspases: initiator,effector, and inflammatory[LAWEN2003]. Initiator caspases-2,-8,-9,and -10 couple various death signals to the core apoptotic machinery. These procaspases have large prodomains that contain sequence motifs that are homologous to motifs present in a number of signalling molecules. Motifs such as the caspase activation recruitment domain (CARD) and death effector domain (DED) mediate activation of initiator procaspases into active proteases[YANG1998]. Effector caspases-3, -6, and -7, have short prodomains and are proteolytically activated by upstream initiator caspases. It is the effector caspases that cleave cellular substrates to mediate the morphological aspects of apoptosis. Rather than function in apoptosis, inflammatory caspases play a major role in cytokine maturation[GRUTTER2000].

In addition to disassembling cell structure and preparing the cell for phagocytic digestion, caspases halt cell cycle progression and disable homeostatic and repair systems. In agreement with the notion that caspases execute apoptosis, abrogation of caspase activity through mutation or pharmacological inhibitor will slow down or block

apoptosis[EARNSHAW1999]. Furthermore, gene knockouts of effector caspases -3 and -9 resist apoptosis and develop abnormally[HAKEM1998].

Effector caspases are responsible for executing most of the morphological changes characteristic of apoptosis. Caspases selectively cleave proteins that can lead to inactivation or activation of the target protein. Chromation condensation in the nucleus results from lamin cleavage[RUCHAUD2002]. DNA repair mechanisms are halted when poly(ADP-ribose)polymerase (PARP), a DNA repair enzyme, is cleaved and inactivated[BOULARES1999]. Cellular shape changes are primarily due to inactivation of cytoskeletal proteins such as fodrin and gelsolin[KOTHAKOTA1997]. Cleavage between the negative regulatory subunit and catalytic subunit of p21-associated kinase-2(PAK2)[RUDEL1997] activates the target protein, leading to subsequent membrane blebbing and apoptotic body packaging. Another example of caspase-mediated activation of proteins is the DNA ladder nucleus CAD, which is activated following cleavage of an inhibitory subunit[ENARI1998].

Besides activating effector procaspases, initiator caspases themselves can play an "executioner" role in apoptosis. Caspase-8 can cleave plectin, a protein responsible for crosslinking members of all three filament systems of the cytoskeleton, prior to any other cytoskeletal protein caspase substrates[STEGH2000]. Perhaps the most important caspase-8 substrate is the BH3-only protein BID, capable of bridging extrinsic death signals to core apoptotic machinery[LUO1998]. Following caspase-8 cleavage of cytosolic BID, truncated BID (tBID) translocates and inserts into the mitochondrial outer membrane[WEI2000]. tBID then cooperates with the multidomain proapoptotic Bcl-2 family members BAX[ESKES2000] and BAK[LUO1998] to release mitochondrial pro-apoptotic factors including cytochrome c. Another caspase-8 substrate that is believed to be critical for the progression of apoptosis

is the ER resident BAP31[NG1997]. The presence of crBAP31, a mutant version of BAP31 lacking its caspase recognition sites, prevents membrane fragmentation and cytochrome c release[NGUYEN2000]. These results imply that BAP31 mediates apoptotic cross-talk between the ER and the mitochondrion.

INITIATOR PROCASPASE ACTIVATION

Death receptors are cell surface receptors that transmit apoptotic signals initiated by specific ligands. Death receptors such as Fas receptor and Tumor Necrosis Factor Receptor-1 (TNFR-1) mediate apoptosis through the formation of a death inducing signalling complex (DISC)[MUZIO1998]. Association of Fas ligand with its receptor promotes receptor trimerization and clustering of intracellular death domains(DD)[PETER2003]. This event serves to recruit and oligomerize adaptor proteins such as Fas-associated death domain(FADD)[CHINNAIYAN1996]. FADD is then capable of recruiting an initiator procaspase (-8 or -10) through their prodomain DED. Although procaspase-8 only possesses 1-2% of the activity of the active form, this is sufficient for processing and production of active caspase-8[MUZIO1998]. As mentioned earlier, caspase-8 can cleave BID, generating tBID that translocates to the mitochondria to stimulate cytochrome c release.

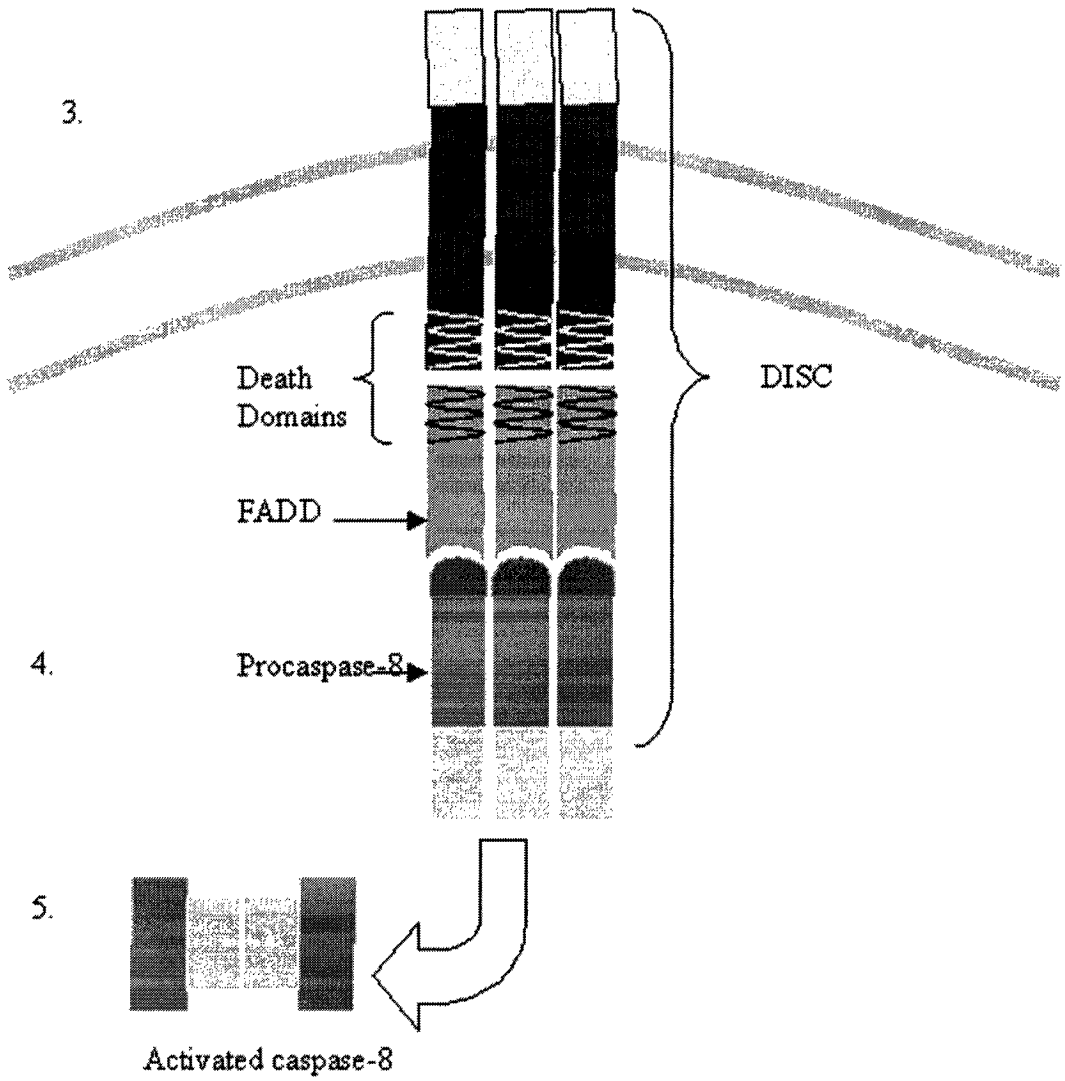

Figure 1.2 – The Death Inducing Signalling Complex (DISC). Ligand binding induces receptor trimerization and intracellular DD clustering, which recruits FADD. FADD then recruits procaspase-8 molecules through their prodomains. Clustering of procaspase-8 leads to activation of mature caspase-8. (Chen M, Wang J. 2002. *Apoptosis*. 7: 313-319)

Released cytochrome c can bind to Apaf-1, the functional homologue of *C.elegans* CED-4[ZOU1997]. In the presence of cytochrome c, WD40 repeats of Apaf-1 molecules are able to associate with each other[CHANG2003]. Apaf-1 oligomerization generates a wheel-like structure that contains seven molecules of Apaf-1, cytochrome c, and ATP[ACEHAN2002].

Oligomerization exposes the CARD domain within Apaf-1, resulting in recruitment of seven procaspase-9 molecules. Although the mechanism of caspase-9 activation is not clear, active caspase-9 is known to cleave and activate the effector caspase-3. The importance of apoptosome formation is highlighted by the observation that cells deficient for cytochrome c[LI2000], apaf-1[YOSHIDA1998], caspase-9[KUIDA1998], or caspase-3[KUIDA1996] exhibit reduced cell death.

During stress-induced apoptosis, caspase-2 is considered to be the apical caspase[SCHWEIZER2003]. Caspase-2 is recruited to a large cytosolic protein complex devoid of Apaf-1 and cytochrome c, suggesting the presence of an Apaf-1-independent cytosolic apoptosome[READ2002].

In addition to being a critical caspase-8 substrate, Bap31 can complex with procaspase-8, a CED-4-like protein, and Bcl-XL[NG1998]. This indicates that procaspase-8 is localized to multiple sites in the cell and depending on localization, each pool of caspase-8 may cleave a distinct set of target proteins. Interestingly, a novel isoform of procaspase-8, procaspase-8L, is selectively recruited to the Bap31 complex through an N-terminal extension (NEX) domain[BRECKENRIDGE2002].

THE BCL-2 FAMILY

The first mammalian homologue for ced-3, Bcl-2, was involved in B-cell lymphomas[VAUX1988]. B cells normally undergo apoptosis following IL-3 withdrawal. Transfected Bcl-2 in these conditions suppressed apoptosis, displaying that tumor progression relies on escaping cell cycle control and prevention of apoptosis. Many signals leading to caspase activation are regulated by the Bcl-2 protein family.

The evolutionarily conserved family of Bcl-2 proteins can be classified into three subfamilies based on function and presence of Bcl-2 Homology (BH) domains: (1) Anti-

apoptotic; (2) Multidomain pro-apoptotic; and (3) BH3-only pro-apoptotic. The "rheostat" hypothesis states that the relative levels of pro- and anti-apoptotic proteins determine a cell's susceptibility to apoptosis[KORSMEYER1995]. This is based on observations that pro-apoptotic members are capable of heterodimerizing with and inactivating the anti-apoptotic members of the Bcl-2 family. This interaction involves the amphipathic helix formed by the BH3 domain of pro-apoptotic Bcl-2 members binding to a hydrophobic groove on the surface of the anti-apoptotic Bcl-2 family members[FESIK2000].

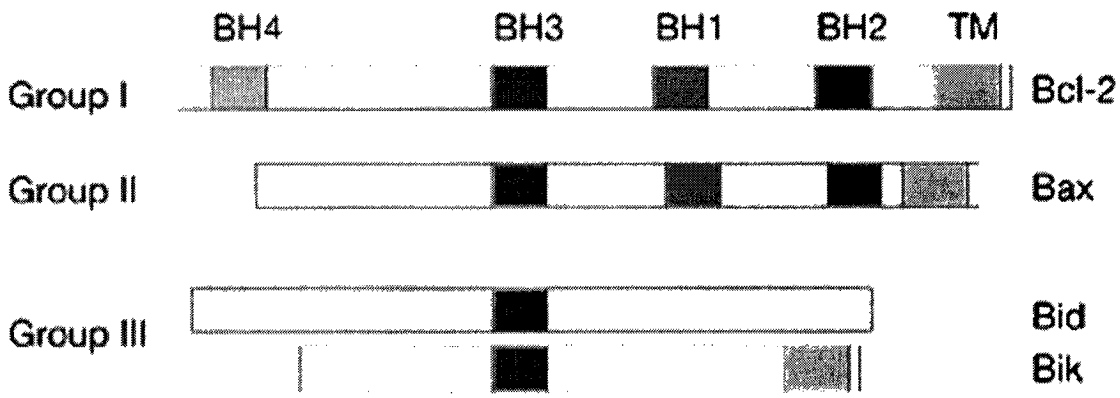

Figure 1.3 – The subfamilies of the Bcl-2 family of proteins. Group 1 consists of anti-apoptotic members while Group II and group III consist of proapoptotic members. Classification is based on function and BH domains. (Adams J, Cory S. *Science*. 281: 1322-1326)

The anti-apoptotic subfamily has all four BH domains. Bcl-2 and Bcl-XL, the two most important anti-apoptotic members of the Bcl-2 family, each possess a C-terminal hydrophobic helix that functions as a membrane targeting domain[NGUYEN1993]. It is believed that Bcl-2 and Bcl-XL are redundant in their capacity to protect cells from apoptosis[CHAO1995]. The importance of Bcl-2 in the apoptotic process is highlighted by

bcl-2 knockout mice surviving embryonic development but eventually exhibiting marked lymphoid apoptosis, melanocyte, neuronal, and intestinal lesions, and terminal kidney disease[VEIS1993].

The multi-domain pro-apoptotic proteins have all but the BH4 domain. Mammalian Bax and Bak promote cell death in spite of the homology to Bcl-2 at BH domain 1-3. It should be mentioned that although Bax and Bak are critical for apoptotic progression in mammals[WEI2001], no ortholog has been identified in *C.elegans*.

The BH3-only pro-apoptotic members, as their name implies, possess only one of the BH domains. Many BH3-only proteins were discovered in protein interaction traps by binding to Bcl-2 or Bcl-XL, indicating that they are expressed in the cell under normal conditions. However, they are present in healthy cells in a dormant form and activated by posttranslational modifications, insuring that inappropriate cell death is not triggered. BIM[PUTHALAKATH1999] and BMF[PUTHALAKATH2001] are normally sequestered to the dynein motor complex and myosin V actin motor complex, respectively. In response to cell detachment (anoikis), these BH3-only proteins translocate to intracellular sites and bind anti-apoptotic Bcl-2 family members. Growth signaling leads to phosphorylation and sequestration of BAD to 14-3-3 scaffold proteins[ZHA1996]. Upon growth-factor withdrawal, BAD is dephosphorylated, which leads to its binding of Bcl-2. Some BH3-only proteins are only expressed under conditions where the cell is damaged in a particular manner. In response to stress signals including DNA damage, the tumor suppressor p53 transcriptionally upregulates many proteins to promote apoptosis, including the BH3-only proteins PUMA[JEFFERS2003], NOXA[ODA2000], and BIK[MATHAI2002]. Since BH3-only proteins are activated in response to many different stimuli, it is believed that BH3-only proteins act as sensors of cell damage and stress[BOUILLET2002]. In

agreement with the notion that BH3-only are important for the initiation of apoptosis, targeted disruption of BH3-only genes in mouse models leads to protection from apoptosis and an increased incidence of tumors. For example, in mice with either PUMA or NOXA disrupted, a diminished apoptotic response is observed when treated with a variety of cytotoxic agents, including ionizing radiation and cytokine deprivation[VILLUNGER2003].

THE MITOCHONDRIA

Mitochondria are considered the 'powerhouses' of the cell, supplying ATP for cellular functions, thereby promoting survival. This organelle also possesses a pro-apoptotic function, capable of coupling various death signals into a common death pathway. This death pathway involves the release of pro-apoptotic factors such as cytochome c into the cytosol. As mentioned above, cytochrome c promotes formation of the apoptosome, which leads to caspase-3 activation and cellular demise. The mitochondria contains several other apoptotic proteins, including the flavoprotein apoptosis inducing factor (AIF)[SUSIN1999], endonuclease G, and second mitochondria-derived activator of caspases Smac/Diablo[VERHAGEN2000]. Similarly to endonuclease G, AIF translocates to the nucleus where it promotes chromatin condensation and DNA fragmentation. Smac/Diablo is responsible for neutralizing the inhibitor of apoptosis proteins (IAP), which normally promote cell survival by inhibiting caspases[WANG1999]. As a consequence of these events, mitochondrial dysfunction occurs. Hallmarks of dysfunction include swelling, altered membrane potential, and production of reactive oxygen species.

The mitochondrial outer membrane (MOM) must be permeabilized in order for proteins to be released. The mechanism by which this occurs, known as the

mitochondrial membrane permeabilization (MMP), remains elusive. However, it is certain that pro-apoptotic and anti-apoptotic members of the Bcl-2 family play a role in the release of these pro-apoptotic factors and the regulation of apoptosome assembly. In general, it is accepted that BH3-only and BAX-like proteins induce release of cytochrome c, whereas Bcl-2 and Bcl-XL inhibit it.

BH3-only proteins promote apoptosis by two mechanisms: inactivation of Bcl-2 and Bcl-XL by direct binding[BOUILLET2002] and modification of multidomain Bcl-2 proapoptotic members[HUANG2000]. Furthermore, BH3-only proteins can be further classified as "activators" or sensitizers"[LETAI2002]. This is based on the finding that the BH3 domains of BID and BIM activate BAX and BAK when added to mitochondria, whereas BH3 domains of BAD and BIK cannot. Instead, BH3 domains of BAD and BIK bind the anti-apoptotic Bcl-2 members. Thus, lower concentrations of BID-like proteins are required to activate BAX-like proteins since BAD-like proteins sequester the anti-apoptotic Bcl-2 proteins. Activation of BID to tBID(truncated BID) by caspase-8 cleavage results in translocation and insertion into the MOM[LUO1998]. Activated tBID becomes an alkali-resistant, integral membrane protein that interacts with BAX and BAK[RUFFOLO2000]. The activation event of BAX and BAK involves a conformational change and their subsequent homooligomerization[RUFFOLO2003]. BAX and BAK are believed to be key participants in the release of cytochrome c since cells lacking both of these proteins do not release cytochrome c in response to diverse apoptotic stimuli[WEI2001].

There are several models that attempt to explain how MMP proceeds following BAX and BAK activation. The first model postulates that BAX and BAK form homomultimeric pores[SAITO2000]. In addition, BAX, Bcl-2, and Bcl-XL can form ion channels in artificial membranes [ANTONSSON1997]. However, there is no data to support this

model in vivo. The second model suggests that Bcl-2 family stabilize or destabilize an existing channel known as the permeability transition pore (PTP), which forms across contact sites between the inner and outer mitochondrial membranes[CROMPTON1999] . Core components of the PTP include the voltage dependent anion channel (VDAC) in the MOM, the adenine nucleotide translocator (ANT) in the IMS, and cyclophilin D in the mitochondrial matrix. The PTP is most likely involved since cyclosporin A, an inhibitor of the PTP, inhibits cytochrome c release[ZAMZAMI2001]. BAX is able to induce a "full" permeability transition which is accompanied by mitochondrial swelling and depolarization or a transient permeability transition lacking swelling and depolarization but inducing release of matrix-localized calcein[PASTORINO1999]. A transient opening of the PTP is more likely since mitochondrial production of ATP is still required for execution of apoptosis. Moreover, oligomeric BAX releases cytochrome c in absence of a permeability transition while monomeric BAX releases cytochrome c in presence of a permeability transition[GOGVADZE2001]. Since it is known that tBID releases cytochrome c without swelling[KLUCK1999], these observations suggest that BAX and BAK cooperate with components of the PTP to induce a transient opening and that mitochondrial swelling and depolarization are secondary events. Indeed, these two events were shown to be caspase dependent and not required for initiation of MMP[GOLDSTEIN2000].

In order for proteins to cross the MOM into the cytoplasm, mitochondrial remodeling events are required[SCORRANO2002]. Since only 15-20% of the total cytochrome c is in the intermembrane space (IMS)[BERNARDI1981, the cristae stores of cytochrome c must also be mobilized in order to be released. Cytochrome c in cristae is separated from the IMS by narrow cristae junctions. These junctions are opened when cristae become fused, making cytochrome c available for release into the IMS.

Under normal conditions, the shape of the mitochondrial network results from a balance between fusion and fission that is regulated by dynamins[YAFFE1999]. A dominant negative mutant of Drp1, a dynamin related protein involved in mitochondrial fission, can block fragmentation of the mitochondria, MMP[FRANK2001], and apoptosis.

THE ENDOPLASMIC RETICULUM

The endoplasmic reticulum(ER) is the site of protein synthesis, folding, and trafficking as well as acting as a cell stress sensor. The role of the ER, until recently, was considered to sensitize the mitochondria to death signals. However, the ER, like the mitochondria, is now believed to play a role in the initial stages of the intrinsic pathway. It is now known that Bcl-2 family members reside at the ER and that procaspase activation can occur at the ER, indicating that the ER has components of the intrinsic machinery to receive and transmit apoptotic signals. The ER can participate in apoptosis in at least two ways: the unfolded protein response (UPR) and Ca^{2+} signaling.

The UPR is activated when the capacity of the ER to properly fold proteins has been compromised. In general, this involves halting the translation machinery as well as upregulating ER chaperones and other components of the secretory pathway[TRAVERS2000]. Three transmembrane proteins Ire1, Atf6, and Perk are the main sensors of unfolded protein accumulation[PATIL2001]. Accumulated unfolded proteins bind the ER chaperone BiP, disrupting its interactions with two other proteins, Ire1 and PERK. Ire1 and PERK, normally inactive when bound to BiP, each homo-oligomerize and auto-transphosphorylate within their serine/threonine kinase domains[KATAYAMA1999]. One isoform of Ire1 possess ribonuclease activity and cleaves 28S rRNA, inhibiting translation[IWAWAKI2001]. Ire1 also possesses endonuclease activity that is required to cleave a short segment from X-box binding protein (XBP-1), generating a bZIP

transcription factor that upregulates ER chaperone transcription[SHEN2001]. PERK phosphorylates the translation initiation factor eIF2α, leading to translation downregulation[HARDING1999]. ATF6 is cleaved during the ER stress response, generating a cytosolic fragment that translocates to the nucleus and functions as a basic leucine zipper transcription factor[MCCULLOUGH2001].

These events give the cell a chance to restore proper ER function. However, if the damage is too great, apoptosis is induced. ER stress-induced apoptosis can by studied by blocking N-linked glcosylation (tunicamycin, TN), inhibiting ER to Golgi transport (brefeldin A, BFA), disrupt disulfide bonds (dithiothreitol, DTT), or perturbing ER Ca^{2+} stores (thapsigargin, TG).

In addition to being a fundamental second messenger, Ca^{2+} also plays a role in apoptosis. It is known that the ER possesses the cell's largest store of Ca^{2+} [POZZAN1994]. Ca^{2+} in the ER is free or bound to lumenal proteins such as calreticulin(CRT) and calnexin(CNX). Activation of the ER Ca^{2+} release channels, inositol 1,4,5-triphosphate receptor (IP_3R) and ryanodine receptor (RyR), decreases $[Ca^{2+}]er$ and thereby increases $[Ca^{2+}]c$ whereas the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) increases $[Ca^{2+}]er$. Fluctuations in ER steady state Ca^{2+} levels as well as Ca^{2+} release can be apoptotic. For example, IP_3R-deficient lymphocytes are resistant to a variety of death signals[JAYARAMAN1997]. Conversely, SERCA inhibition by TG induces apoptosis[MA1999]. ER release of Ca^{2+} generates spikes and waves whose frequency and amplitude are sensed by Ca^{2+} -sensitive enzymes[THOMENIUS2003]. The Ca^{2+} -sensitive cysteine protease calpain has been observed to cleave procaspase-12[NAKAGAWA2000]. Caspase-12 is activated specifically in response to ER stress, and neurons lacking caspase-12 resist apoptosis by TN. This finding indicates that the ER is directly connected to its own caspase cascade.

Caspase-12 activation is also directly linked to Ire1 signaling. Ire1 can recruit Traf2, which interacts with and activates caspase-12[YONEDA2001]. In addition, ER stress can induce translocation of caspase-7 to procaspase-12 and activate caspase-12[RAO2001]. Calpain can also activate the serine/threonine phosphatase calcineurin by cleaving the calcineurin-binding domain of its inhibitor, cain[KIM2002]. Once activated, calcineurin dephosphorylates the BH3-only protein BAD, which allows BAD to translocate to the mitochondria and antagonize Bcl-XL[WANG1999]. Similarly to caspases, calpain has also been reported to cleave BID to tBID[MANDIC2002], as well as Bcl-2 and Bcl-XL.

Ca^{2+} has the capacity of impinging on the mitochondrial apoptotic machinery. IP_3R-mediated Ca^{2+} spikes could affect MMP, because mitochondrial Ca^{2+} uptake sites are proximal to IP_3R-containing ER[SZALAI1999]. Similar to their role in mitochondrial release of cytochrome c, BAX and BAK appear to be essential for redistribution of Ca^{2+} from the ER to the mitochondria. BAX, BAK double-null cells have decreased $[Ca^{2+}]er$[SCORRANO2003], which results in a decreased Ca^{2+} uptake by mitochondria and resistance to apoptosis. SERCA overexpression restores sensitivity to apoptosis. In addition, caspase-12 processing is abrogated in BAX, BAK double-null cells and can be induced by ER-targeted BAK[ZONG2003]. Furthermore, BAX and BAK can release Ca^{2+} from the ER to the mitochondria and induce cytochrome c release[NUTT2002]. This indicates BAX and BAK at the ER have a distinct role from its counterparts in the mitochondria.

Similarily to its role at the mitochondria, Bcl-2 at the ER can antagonize BAX and BAK function. ER-localized Bcl-2 can enhance ER membrane permeability by regulating the IP_3R and the SERCA[BERRIDGE1998]. Bcl-2 decreases steady-state free $[Ca^{2+}]er$, and this drop protects cells from cytochrome c release in certain signaling pathways[PINTON2001]. For example, ER-targeted Bcl-cb5 prevents apoptosis by TN and

inhibits cytochrome c release[THOMENIUS2003]. This indicates that crosstalk between ER and mitochondria can be interrupted by ER-localized Bcl-2[HACKI2000]. The ER also harbors regulators such as RTN-XS which are capable of reducing the anti-apoptotic activity of Bcl-2 and Bcl-XL[TAGAMI2000].

In contrast to most BH3-only proteins that localize to the mitochondria, BIK resides at the ER and participates in ER to mitochondria crosstalk. BIK is capable of initiating cytochrome c release in the absence of caspase activity[GERMAIN2002]. BIK may accomplish this by inducing BAX and BAK oligomerization in the ER and/or influencing Ca^{2+} release.

A strong argument can be made for an initiating role in apoptosis based on studies of BAP31. As mentioned above, crBAP31 inhibits several apoptotic events at the mitochondria, in the presence of active caspases. This indicates that the ER exerts a restraint upon the apoptotic machinery. BAP31 is found in complex with many proteins involved in apoptosis, including procaspase-8L, A4[WANG2003], and Bcl-2. Procaspase-8L, along with procaspase-12, represent two initiator caspases that are localized to the ER, supporting a role for ER in initiation of apoptosis. A4 is a putative ion channel and it has been proposed that BAP31-A4 might regulate Ca^{2+} release. Bcl-2 can neutralize potential proapoptotic activity of BAP31 by inhibiting caspase cleavage to p20. As mentioned above, Drp1 recruitment to mitochondria is involved in fragmentation and fission.

p20 induces ER release of Ca^{2+}, mitochondrial uptake of Ca^{2+}, Drp1 recruitment, and fragmentation and fission of mitochondrial network[BRECKENRIDGE2003].

The "two-hit" model proposes that an ER Ca^{2+} signals cooperates with BH3-only protein to promote cytochrome c release[SCORRANO2002]. For example, p20 may cooperate

with tBID to induce cristae remodelling and cytochrome c release. In a physiologial setting, BH3-only proteins may be suboptimal and their function may require costimulating action from the ER.

P53 ACTIVATION PATHWAYS

Various cell stress signals such as DNA damage and oncogene expression are coupled to the intrinsic apoptotic machinery via the tumour suppressor p53. The importance of p53 is evident since more than 50% of tumours have a p53 gene mutation[HOLLSTEIN1991] and p53 knockout mice show a high incidence of tumour development[DONEHOWER1996]. The ability of p53 functions to control passage through cell cycle and to control apoptosis in response to abnormal proliferative signals and stress including DNA damage is important for its tumour suppressor function.

P53 is a nuclear phosphoprotein that contains (1) an N-terminal acidic transactivating domain; (2) a central DNA-binding domain; (3) homotetramerization domain; and a (4) putative DNA damage recognition domain. Mutations in any of these domains may produce a dominant negative inhibitor of wild type p53[DEVRIES2002].

P53 is a very short-lived protein and is stabilized upon a stress signal[PRIVES1999]. P53 protein levels are under stringent control, its abundance and activity being regulated by many post-translational modifications such as glycosylation, ubiquitination, and sumolyation[MELCHIOR2002]. The amount of p53 protein expressed is determined by rate at which it is degraded, rather than rate at which it is made. Degradation proceeds through ubiquitin-mediated proteolysis. This involves the conjugation of polyubiquitin chains by ubiquitin ligases such as MDM2 to specific residues on p53, and recognition by the proteasome[MOMAND2000]. The proteasome is the major protein-degrading machinery of the cell and is responsible for p53 protein turnover. MDM2, a ring-finger ubiquitin ligase, is

considered to be a major determinant of p53 stability. Stress is sensed by several kinases, including the DNA-dependent kinase ATM, CHK1, and CHK2. ATM phosphorylates CHK1 and CHK2, which in turn phosphorylate p53[SHIEH2000]. In addition, ATM also phosphorylates p53[CANMAN1998] and MDM2[MAYA2001]. Phosphorylation of MDM2 interrups its binding to p53, leading to p53 protein stabilization. p53 promotes cell cycle arrest by transactivating many genes. One critical target gene is the cyclin-dependent kinase inhibitor p21[ELDEIRY1993], which is the means by which p53 induces G1 cell cycle arrest. If DNA repair is successful, then the cell can enter S phase. If the damage is beyond repair, p53 can induce apoptosis by transactivating p53 inducible genes (PIGS).

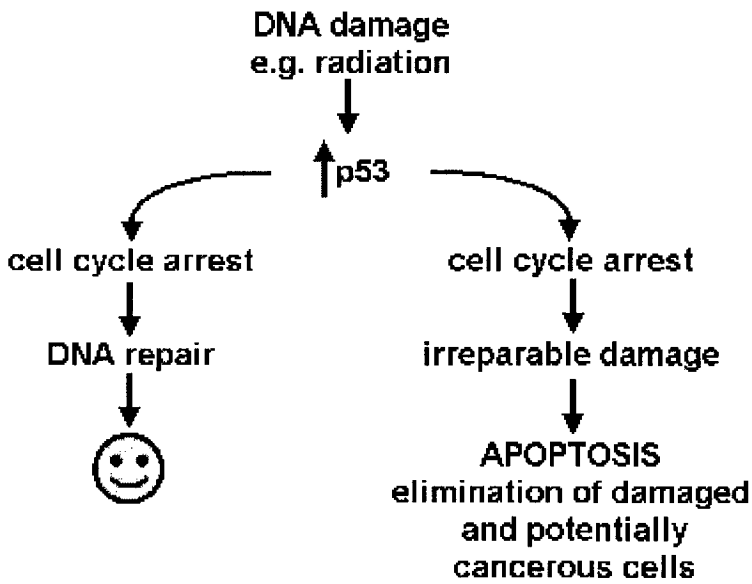

Figure 1.4 – The tumour suppressor p53. In response to DNA damage, the cell increases p53 protein levels. p53 induces cell cycle arrest and if the damage is too great, p53 induces apoptosis. p53 induces these two pathways through its transcription-dependent mechanisms. (de Stanchina et al. *Genes Development.* 12: 2434-2442)

It is known that p53-dependent apoptosis depends on apoptosome formation[SOENGAS1999]. p53 induces mitochondrial dysfunction by activating genes that are involved in mitochondrial-induced apoptosis, including p53AIP1[OKA2000], PIDD[LIN2000] and many Bcl-2 family members. BH3-only proteins NOXA[ODA2000], PUMA[NAKANO2000], and BIK[MATHAI2002] are upregulated as well as the multidomain BAX. Upregulated Bcl-2 members localize to both the ER and the mitochondria to promote apoptosis. P53 mediates apoptosis in a transcription independent manner as well, interacting with Bcl-XL and Bcl-2 at the mitochondria.

Another p53 activation pathway involves oncogene expression [SHERR2000]. Oncogenes such as Early region 1A of Adenovirus (E1A) and c-Myc are capable of activating p53[HERMEKING1994]. Oncogene expression that deregulates the cell cycle either induces apoptosis or sensitizes cells to apoptotic stimuli. When retinoblastoma protein (RB) is bound to E2F, entry into S phase is inhibited[GOTTLIEB1996]. To induce apoptosis, E1A associates with and inactivates RB, allowing p53 accumulation. In addition, E1A can stimulate p14 transcription, which binds to MDM2 and inhibits its activity.

OBJECTIVES

My goal was to investigate the role of BIK during p53-mediated apoptosis by conducting a targeted disruption of the putative BIK ortholog, BLK (BIK-Like-Killer), in mice. Initially, I had to confirm that BLK is indeed the mouse ortholog of BIK. BLK has 43% sequence homology to BIK and an identical BH3 domain, a region that is critical for its proapoptotic activity, making it the strongest candidate mouse ortholog. In human cells, our lab has shown that p53 induces BIK messenger RNA and protein. I will determine if elevated levels of p53 induces expression of BLK messenger RNA and protein levels. Our lab has also shown that BIK localizes to the ER membrane and induces cytochrome c release from this location in human cells, an observation that has not been ascribed to any other BH3-only protein to date. I will perform similar studies with BLK in mouse and if these criteria are met, the homology of BLK to BIK will be confirmed.

Furthermore, since an emerging theme in regulation of apoptosis is intracellular localization, we investigated the localization of BLK and BIK by swapping their transmembrane domains (TMD).

METHODS AND MATERIALS

Cell culture, adenovirus vector infection, and apoptosis assays

Human H1299 lung carcinoma and 293T embryonic kidney cell lines were cultured in a α-MEM medium supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin and penicillin. Mouse RENCA renal carcinoma cell line was cultured in a RPMI medium supplemented with 10% fetal bovine serum and 100μg/ml streptomycin and penicillin. Mouse 3T3 embryo fibroblast and monkey COS-7 kidney cell lines were cultured in a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin and penicillin. Cells were infected at 100 plaque-forming units (PFU)/cell with adenovirus vectors expressing either wild-type p53, reverse tetracycline-controlled transactivator (rtTA), or wild-type Bcl-2. Briefly, cDNAS encoding p53, rtTA, and Bcl-2 were subcloned into a pCA14 variant containing the T-Rex promoter (Invitrogen), which functioned as a shuttle vector[BETT1994]. The cell line for virus rescue was 293T cells stably transfected with pcDNA6/TR (Invitrogen), which expresses the tetR under control of the CMV promoter, and termed 293T-TR. The T-Rex variants were introduced into 293T-TR cells using Lipofectamine Plus (GIBCO BRL) together with pJM17[MCGRORY1988], the adenoviral genomic construct. After recombination in the cells, the virus was produced with T-Rex driven (but silent) cDNAs in place of the E1 region of the virus. Virus plaques were screened by PCR for presence of the correct insert, plaque purified again, then propagated in 293T-TR cells. Virus preparations were made from freeze/thaw lysates and titrations conducted on this same cell line. DEVD-amc caspase activity assay (Upstate Biotechnology) was obtained by treating cells with 50mM Hepes pH7.4, 1% Triton X-100, 5mM EDTA, and 2mM dithiothreitol, and incubating the extract with 50μM AcDEVD-amc for 30 min at 37o. Fluorescence in the linear range of DEVDase activity was determined using a plate reader (Tecan).

Antibodies and immunoblots

The following antibodies were utilized: mouse anti-FLAG (Sigma), mouse monoclonal anti-p53 (PharMingen), goat polyclonal anti-BIK(Santa-Cruz Biotechnology, CA, USA), rabbit polyclonal BLK (Sigma), rabbit polyclonal anti-p21(Santa-Cruz Biotechnology, CA, USA), rabbit anti-calnexin (gift from JJ Bergeron). Rabbit polyclonal antibody against human TOM20 was generated exactly as described by Goping *et al.* (1988). Alexa 594 and 488 conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA). For immunoblot analysis, aliquots of cell extracts containing equivalent amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and the blots incubated with primary antibody. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase, and visualized by enhanced chemiluminescence (PerkinElmer LifeSciences).

Cloning and mutagenesis

BLK cDNA was cloned by PCR using an oligonucleotide-dT mouse embryo cDNA library. The 5' sense primer contained a sequence encoding a FLAG epitope tag, which preceded the BLK coding region. The primers used were 5'-CGGGATCCATGGACTACAAAGAC-GATGACGATAAATCGGGAGGCGAGACTTATGGC-3' and 5'-GGAATTCTCACTGA-AGCTGCAAATACCA-3'. An analagous procedure was used to create FLAG-BIK$^{\mathbf{MATHAI2002}}$. Standard PCR site directed mutagenesis was performed to convert BLK codon 55 from Leu to Gly using the above primers in addition to 5'-**SENSE**-3' and 5'-**ANTISENSE**-3'. PCR fragments were cloned into pcDNA3 cloning vector (Invitrogen, Carlsbad, CA, USA) and recombinant plasmids introduced into *E.coli* XL1. Authenticity and insert orientation of purified plasmid were confirmed by DNA sequencing. An analogous procedure was used to create FLAG-BIK(L61G)$^{\mathbf{MATHAI2002}}$. BLK cytosolic portion fused to the transmembrane domain of BIK (FLAG-

BLK-BIKTM) and BIK cytosolic portion fused to the transmembrane domain of BLK (FLAG-BIK-BLKTM) were created using the above primers in addition to5'-ACTCCTGGCGCCTGGGTGTCATGCGAACAGGTGCTG-CTGGCG-3' and 5'-AACCCCGG-GTCCTGGGTGTCCCCTGACCAGGACCCT-GGGCAG-3' respectively. BLK and BIK transmembrane (TM) domain cDNA were fused to green fluorescent protein (GFP) by cloning into p-EGFP C1 vector. The primers used to obtain BLK TM cDNA were 5'-CGGAAT-TCTTGGGTGTCACCTGACCAGG-3' and 5'-CGGGATCCTCACTGAAGCT-GCAAAT-3'. The primers used to obtain BIK TM cDNA were 5'-CGGAAT-TCTTGGGTGTCCTGCGAAC-3' and 5'-CGGGATCCTCACTTGA-GCAGCAGG-3'.

Transient transfections

293T and 3T3 cells were grown to approximately 50% confluency in 6-well cell culture dishes. 0.8μg of pcDNA3 plasmids encoding FLAG-BLK, FLAG-BIK, FLAG-BLK (L55G), or FLAG-BIK(L61G) were transfected using Lipofectamine Plus Reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's instruction. zVAD-fmk (50μM final concentration)(Enzyme System Products, Dublin, CA, USA) or vehicle alone were added to appropriate samples 3 h later. Cells were collected 24 h and extracts analyzed.

Northern blots

Total RNA was collected from RENCA cells using TRIzol Reagent as directed by the manufacturer (GIBCO BRL, Gaithersburg, MD, USA). RNA was quantitated by optical density and 25 μg used for Northern analysis. The probe was generated by digesting 10 μg of the FLAG-BLK pcDNA3 plasmid with *Eco*R1 and *Bam*H1. The released FLAG-BLK cDNA insert was resolved on a 1% agarose gel, the fragment isolated, purified, and labeled using an Oligolabeling Kit 9 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in the presence of α^{32}P-dCTP, according to the manufacturer's protocol. The northern blot was stripped and probed

with an actin cDNA probe.

Immunofluorescence

3T3 cells were grown to a confluency of 50% in 24-well dishes on coverslips. The cells were transfected, using Lipofectamine PLUS (Invitrogen), with vectors encoding FLAG-BLK, FLAG-BIK, FLAG-BLKBIK, FLAG-BIKBLK. In addition, zVAD-fmk (50μM final concentration) was added to all cells. 20 h later, the cells are fixed with 4% paraformaldehyde and analyzed by double-label immunofluorescence. Cells were visualized with a Zeiss 510 confocal microscope and images captured and overlaid with the accompanying software.

RESULTS AND DISCUSSION

The Importance of BIK in p53-Mediated Apoptosis generates an Interest in BLK

Human BIK is the founding member of the BH3-only subclass of the Bcl-2 family of proteins. In contrast to other BH3-only proteins which exert their function at the mitochondria, BIK appears to function at the endoplasmic reticulum[GERMAIN2002]. In addition, whereas most BH3-only proteins are expressed under normal conditions, BIK is only expressed upon apoptotic signaling by the tumor suppressor p53[MATHAI2002]. Interestingly, knockdown of BIK protein during p53-mediated apoptosis by RNA interference significantly suppressed apoptosis, suggesting BIK is an essential effector of p53-mediated apoptosis[MATHAI2003]. A targeted disruption of the putative BIK mouse homologue BLK would confirm the importance of BIK in p53-mediated apoptosis. However, confirmation that BLK is indeed the homologue of BIK was required.

The Gene Structures of BLK and BIK are Similar

Genetic analysis has established that the human *Bik* gene is localized to chromosome 22 at region q13.3[VERMA2000]. In agreement with BLK being the mouse homolog of BIK, the mouse *Blk* gene has been mapped to the mouse chromosome 15 syntenic region[AMANNA2001]. In addition, Figure 1A illustrates that the gene structures of BLK and BIK are similar. Each gene contains five exons and four introns of similar size. Both genes also exhibit similar functional organization, containing their start sites, BH3 domain, and stop sites in exons 2, 3, and 5 respectively. The transcription start site for both genes is located approximately 14kb from the translation start site. The 5' untranslated region (UTR) is considered to be unusually long, suggesting an additional

level of regulation exists within these sequences[VERMA2000].

BLK was identified by searching GenBank for sequences that encode the BH3 domain[HEGDE1998]. It is worthwhile to mention that performing a BLAST search of the mouse genome using the BIK sequence yields the BLK sequence only. While BLK and BIK protein share only 44% identity, Figure 1B illustrates that they do possess identical BH3 domains, which are responsible for the killing activity of these proteins.

During cloning and sequencing of BLK, a 24bp insertion not present in the sequence reported by Hegde et al.(Accession# AF048838) was found between the start site and BH3 domain(Accession# BC003732). This corresponds to an 8 amino acid insert, producing a 158 amino acid protein in contrast to the 150 amino acid protein observed by Hegde et al. In fact, the protein alignment presented in Figure 1B indicates that the presence of the insert creates a more favorable alignment for BLK with BIK. Closer inspection of the two sequences also revealed that the BLK^{INSERT} sequence also contains an additional 400bp insertion in its 5' UTR.

BLK and BIK Induction of Apoptosis Requires A Functional BH3 Domain and Caspases

To demonstrate the apoptotic activity of BLK and BIK, human 293T embryonic kidney and mouse 3T3 embryonic fibroblast cell lines were transfected with plasmids encoding BIK, BIK(L61G), BLK, BLK(L55G), in the presence of absence of the pan caspase inhibitor, zVAD-fmk. BIK (L61G) and BLK(L55G) have a conserved leucine residue in the BH3 domain mutated to glycine, preventing BH3-dependent apoptosis. After 24 h,

cell extracts were prepared and their ability to hydrolyze the fluorogenic caspase substrate DEVD-amc was measured. Figure 2 indicates that BLK and BIK can induce apoptosis in both human (293T) and mouse (3T3) cell lines. As expected, the ability of BLK and BIK to induce apoptosis relies on a functional BH3 domain since a point mutation in the BH3 domain of either protein abrogates apoptosis. Furthermore, presence of zVAD-fmk inhibits the ability of BLK and BIK to induce apoptosis, confirming that they cooperate with downstream caspases to execute apoptosis.

The Sensitivity of the BLK Antibody is Sufficient to Detect BLK Protein Function

Previous studies in our laboratory indicate that BIK protein is turned over rapidly[MATHAI2002]. This is based on several observations including (1) E1A induced expression of BIK is detectable at much higher levels in the presence of Bcl-2; (2) mutant BIK, not wild type BIK, is detected following transient transfection; and (3) wild type BIK is detectable in the presence of zVAD-fmk following transient transfection. All three observations suggest that the potent apoptotic activity of BIK prevents its own ongoing synthesis during apoptosis. In this sense, BIK resembles p53 in that it is very unstable and its protein levels are monitored strictly.

To determine (1) if BLK is an unstable protein; and (2) if a commercial BLK antibody is sensitive enough to study BLK protein function, MEFs were transfected with plasmids encoding FLAG-BIK or FLAG-BLK in the presence or absence of zVAD-fmk. After 24 h, cell extracts were prepared, equivalent amounts of proteins, resolved by SDS-PAGE and immunoblotted with antibodies against FLAG and BLK. The upper panel in Figure 3

shows that transfected BLK expression, like BIK, is enhanced in the presence of zVAD-fmk. The bottom panel in Figure 3 establishes that the sensitivity of the BLK antibody is of the same order of sensitivity of the FLAG antibody, therefore sufficient to study BLK protein function.

Divergent Signaling: p53 does Not Induce BLK mRNA and Protein

BIK was identified as an upregulated transcript during apoptosis induced by the oncogene E1A[MATHAI2002]. E1A can induce apoptosis only in the presence of p53 and p53 is required for induction of BIK. To determine if BLK mRNA is regulated in the same manner as BIK mRNA, mouse renal carcinoma (RENCA) cells were infected with adenovirus vector expressing p53, followed by RNA extraction and Northern blot analysis. Figure 4 shows that BLK mRNA is not upregulated by p53, in contrast to BIK mRNA. Furthermore, whereas the presence of Bcl-2 results in higher levels of BIK mRNA, levels of BLK mRNA are unaffected by adenoviral expression of Bcl-2. It is important to note that BLK mRNA was detectable in the absence of p53, whereas BIK mRNA is barely detectable.

Nevertheless, even though BLK and BIK behave differently at the mRNA level, they may still act similarly at the protein level. It is accepted that the proteasome pathway is important for turnover of many regulatory proteins[KURLAND2001]. Although p53 is a well-known target of the proteasome, BIK has also been identified as a proteasomal substrate[MARSHANSKY2001]. In fact, proteasomal inhibitors are potent apoptosis-inducing agents[MACLAREN2001], presumably by stabilizing proapoptotic proteins such as BIK. The

peptide aldehyde lactacystin is a commonly used proteasomal inhibitor and has a high specificity for the proteasome[ALMOND2002].

To assess if BLK protein levels are regulated by p53, RENCA cells were infected with adenovirus vector expressing p53. The infection was done in the presence or absence of either Bcl-2 or lactacystin in order to facilitate detection of BLK protein. After 24 h, cell extracts were prepared and protein extracts were analyzed by SDS-PAGE and immunoblotting. Figure 5 illustrates that p53 expression upregulates p21, but not BLK. Furthermore, presence of Bcl-2 or lactacystin does not allow for detection of BLK protein.

Lack of BLK induction by p53 was very unexpected. To confirm that BLK was not upregulated by p53, several cell lines were tested. All the cell lines described in Table 1 were treated to undergo p53-mediated apoptosis, but none exhibit BLK upregulation at the mRNA or protein level.

Cell Line	*Origin*	*Type*
RENCA	Murine Renal Carcinoma	P53-null(mutation)
3T3	Murine Embyronic Fibroblast	P53-wild type
9D5/10E	Murine Erythroleukemia	P53-temperature sensitive allele
p53KO	Murine	P53-targeted gene disruption
10(1)	Murine Embyronic Fibroblast	P53-deficient

Table 1 – Cell lines tested for BLK upregulation by p53.

In addition, some of these cell lines were induced to commit apoptosis by agents other than p53, including E1A and several ER stress agents such as tunicamycin and thapsigargin (data not shown). However, no evidence for BLK upregulation was observed.

Surprisingly, endogeneous BLK protein was never detected. Any research performed so far on BLK has been done by BLK cDNA transfection. There exists the possibility that p53 does induce BLK but (1) it is degraded through pathways independent of the proteasome or Bcl-2; or (2) levels induced are still below the detection threshold of BLK antibody and immunoblotting. An attempt to address these possibilities might allow for BLK detection.

In spite of gene similarities and BH3 identity, BLK appears to be regulated differently than the human orthologue, BIK.

Divergent Localization: BLK Localizes Primarily to the Mitochondria

Cellular distribution of BIK and BLK was analyzed by double-label confocal immunofluorescence microscopy. 3T3 cells were transfected with either plasmid encoding either FLAG-BIK or FLAG-BLK in the presence of zVAD-fmk to preserve cellular and organelle integrity. Figure 6A confirms that BIK localizes primarily to the ER, as previously reported[MATHAI2002]. BIK colocalizes strongly with the ER marker calnexin as both localize to a reticular network outside the mitochondria. Figure 6B shows that BLK does not share this localization, instead colocalizing with TOM20 at the mitochondrial outer membrane.

A recent study defines a targeting sequence for targeting Bcl-2 family members to the mitochondrial outer membrane[KAUFMANN2003]. In the study, extensive mutagenesis revealed that when the transmembrane domain is flanked by at least two basic amino acids at both ends, it preferentially targeted mitochondria.

Bcl-2 Family Member	***Transmembrane Domain***
BIK	WVSCEQ*VLLALLLLLALLLPLLSGGLHLLL***K**
BLK	WVSPDQDPGQ*LFPMVLLVFLLLGGAWYL*QLQ
BCL-2	PLFDFSWLSL**K***TLLSLALVGACITLGAYLG*H**K**
BCL-XL	ES**RK**GQE**R**FN**R***WFLTGMTVAGVVLLGSLFS***RK**

Table 2 – Transmembrane Domains of Bcl-2 Family Members. Transmembrane Domains are italicized. Basic amino acids are in bold type.

From Table 2, it is clear that BCL-XL would localize primarily to the mitocondrial outer membrane whereas BCL-2 would localize to multiple locations in the cell, implying non-redundant roles for these two proteins. According to the targeting model, neither BIK nor BLK should localize to the mitochondria. However, this model has notable exceptions: for example, BAX and BAK contain basic amino acids at only one end, but localize primarily to the mitochondria.

The Transmembrane Domains of BLK and BIK Localize to the Endoplasmic Reticulum

To gain insight in the divergent localization of BIK and BLK, transmembrane swaps were constructed and analyzed by double-label confocal immunofluorescence microscopy. BIKBLK, the N-terminal portion of BIK fused to the transmembrane domain of BLK, localized preferentially to the endoplasmic reticulum similarily to BLKBIK (N-terminal portion of BLK fused to transmembrane domain of BIK), shown in Figures 6C and 6D.

Although this result supported the targeting model, it did not fit with the observation that BLK targets to the mitochondrial outer membrane. Enhanced Green Fluorescent Protein (EGFP) was then fused to the transmembrane domains of BIK and BLK. Figure 6E illustrates that both EGFP-BIKTM and EGFP-BLKTM colocalize with calnexin at the endoplasmic reticulum.

Construct	***Localization***
BIK	Endoplasmic reticulum
BLK	Mitochondria
BIKBLK	Endoplasmic reticulum
BLKBIK	Endoplasmic reticulum
EGFP-BIKTM	Endoplasmic reticulum
EGFP-BLKTM	Endoplasmic reticulum

Table 3 – Localization of Various BH3-only fusion proteins.

The immunophilin FKBP38 specifically interacts with Bcl-2 and Bcl-XL[SHIRANE2003]. Alteration of FKBP expression has a strong influence on BCL-2 and BCL-XL targeting, overcoming inherent targeting sequences within BCL-2 and BCL-XL. This suggests that the transmembrane domain of a protein is not necessarily the determining factor in localization. It is possible that the N-terminal portion of BLK plays a role in targeting by either cooperating with its transmembrane domain or a binding partner similar to

FKBP38. However, the N-terminal portion of BLK cannot force the BIK transmembrane domain, implying that the BIK transmembrane domain is "stronger" than the BLK transmembrane domain.

SUMMARY

Several BH3-only proteins are regulated by the tumor suppressor p53, which is specifically activated by DNA damage or oncogenic signals that lead to cancer. Many p53-induced pro-apoptotic proteins target the mitochondria, leading to mitochondrial dysfunction and cytochrome c release. Our lab, however, has identified a BH3-only protein BIK, upregulated by p53, that is able to mediate cytochrome c release from mitochondria from a location in the endoplasmic reticulum (ER). This suggests that BIK is an important mediator of p53-mediated apoptosis and that signals emanating from the ER regulate mitochondrial dysfunction on this cell death pathway. BLK has 43% sequence homology to BIK and an identical BH3 domain, a region that is critical for its proapoptotic activity, making it the strongest candidate mouse orthologue. In addition, the gene structures of these two proteins are similar in functional organization. BLK and BIK activate apoptosis in similar fashion in both human and mouse cell lines, dependent on the presence of a functional BH3 domain and active caspases. BLK, like BIK, is very unstable and can be stabilized by addition of pan caspase inhibitor, zVAD-fmk. In contrast to BIK, BLK is not upregulated by p53 at the RNA or protein level in several mouse cell line systems. BLK localizes to the mitochondria, not to the endoplasmic reticulum where BIK resides. To further investigate the localization of BLK and BIK, the transmembrane domains of these proteins were swapped. BLKBIK and BIKBLK both localized to the endoplasmic reticulum. Enhanced Green Fluorescent Protein fused to the transmembrane domains of BLK and BIK also localized to the endoplasmic reticulum. The mitochondrial localization of BLK despite its transmembrane domain preference for the endoplasmic reticulum suggests that the N-terminal portion of BLK

plays a role in targeting. Collectively, these results argue that BLK is not the mouse homologue of BIK.

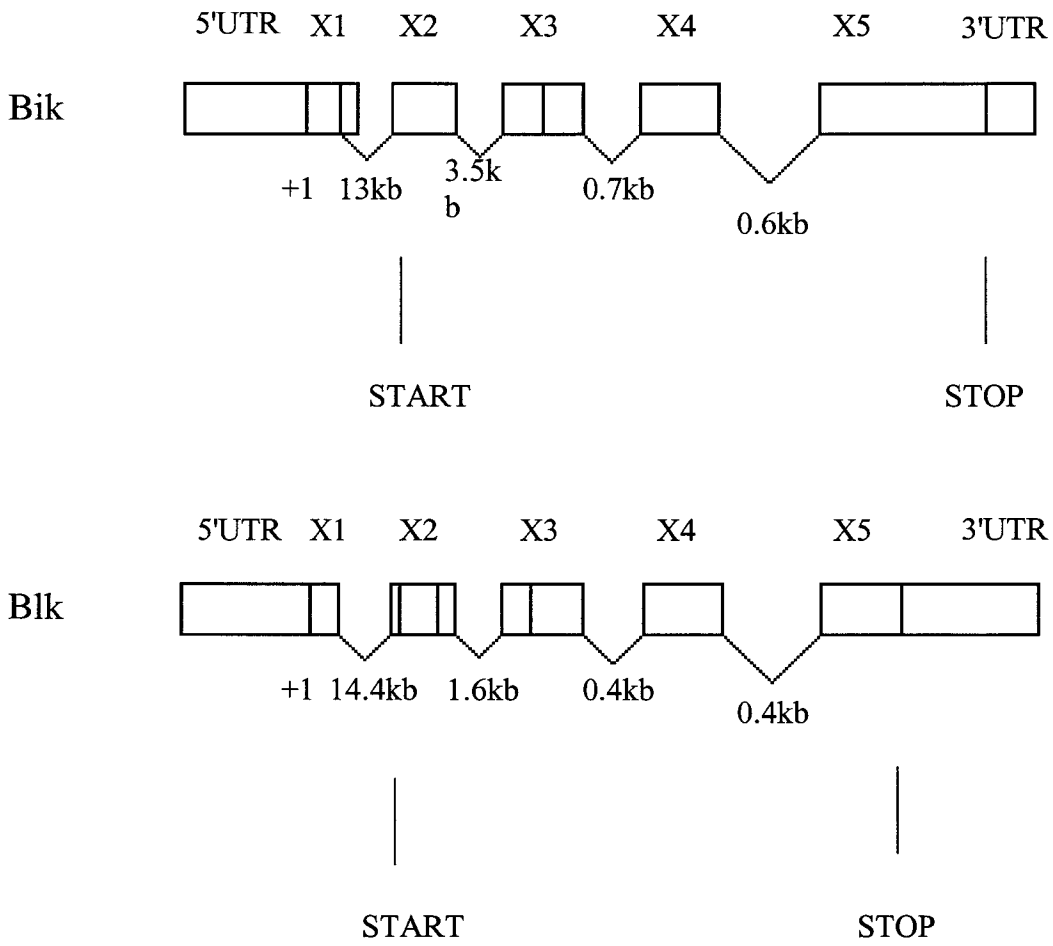

Figure 1A – *Schematic representation of genomic structures of the human Bik and mouse Blk genes.* X1 – X5 represents Exons 1-5; UnTranslated Region (UTR); +1 represents transcriptional start site.

```
BIK          MSEVRPLSRDILMETLLYEQLLEPPTM-EVLGMTD--SEEDLDPMEDFDSLECMEGSDALALRLACIGDEMD
BLK^INSERT   MSEARLMARDVI-KTVPHDQVPQPPVASETPSMKEPVAGENLSPVRDVDLMECVEGRNQVALRLACIGDEMD
BLK          MSEARLMARDVI-KTVPHDQVPQPPVASETPSMKEPV--------RDVDLMECVEGRNQVALRLACIGDEMD

BIK          VSLRAPRLAQLSEVAMHSLGLAFIYDQTEDIRDVLRSFMDGFTTLKENIMRFWRSPNPGSWVSCEQVLLALLL
BLK^INSERT   LCLRSPRLVQLPGIAIHRL--AVTYSRT-GVRGIFRSLIRSLTNLRENIWS-WRVLTPGAWVSPDQDPGQLFP
BLK          LCLRSPRLVQLPGIAIHRL--AVTYSRT-GVRGIFRSLIRSLTNLRENIWS-WRVLTPGAWVSPDQDPGQLFP

BIK          LLALLLPLLSGGLHLLLK
BLK^INSERT   MVLLVFLLLGGAWYLQLQ
BLK          MVLLVFLLLGGAWYLQLQ
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Figure 1B - *Protein Alignment*. Schematic comparing human protein BIK and the mouse proteins BLK and $BLK^{REPORTED}$. The sequences in bold represent the BH3 domains of the three proteins. The underlined sequence in BLK represents an insertion.

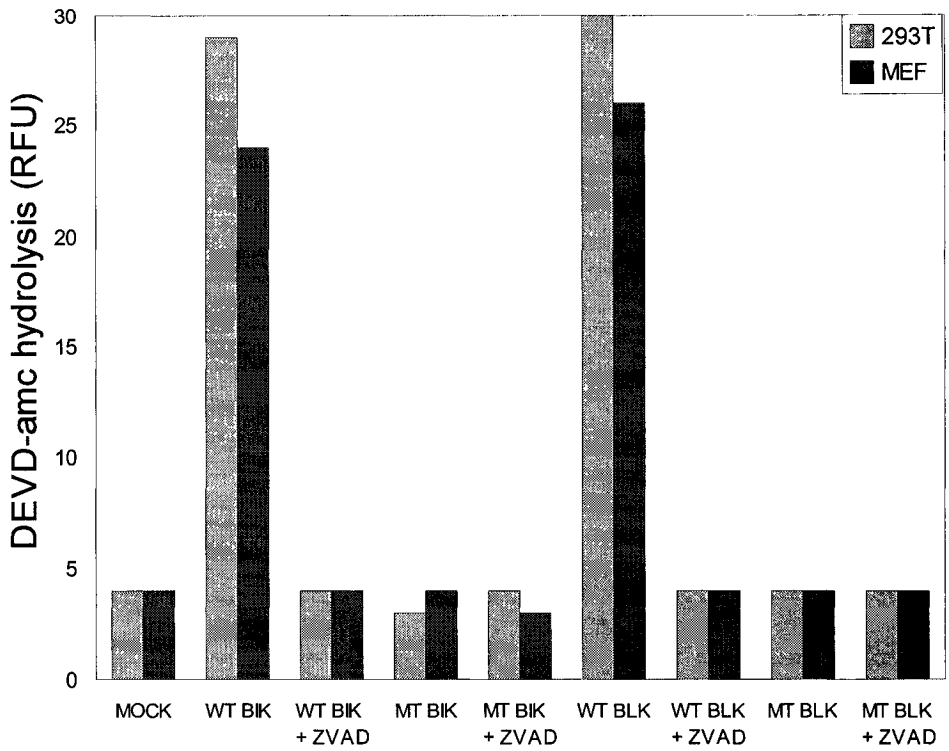

Figure 2 – *BIK and BLK cDNA are proapoptotic.* Transient transfection of 293T and MEF cells with pcDNA3 plasmids encoding BIK, BIK (L61G), BLK, BLK(L55G), in the presence or absence of 50 μM zVAD-fmk. After 24 h, cell extracts were prepared and their ability to hydrolyze the fluorogenic caspase substrate DEVD-amc was measured.

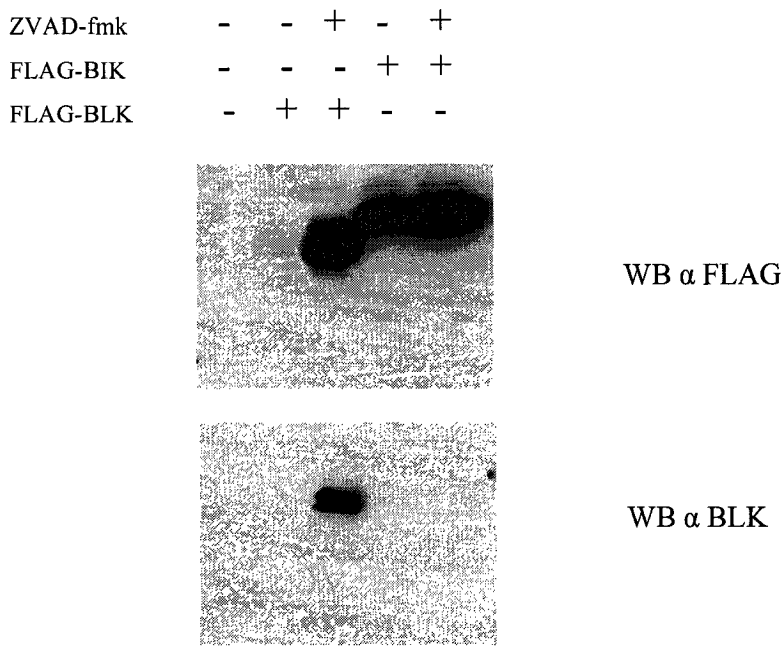

Figure 3 – *BLK antibody sensitivity is similar to FLAG antibody sensitivity.* Transient transfection of MEF cells with pcDNA3 plasmids encoding FLAG-BIK or FLAG-BLK in the presence or absence of 50 μM zVAD-fmk. After 24 h, cell extracts were prepared and and aliquots containing equivalent amounts of proteins were resolved by SDS-PAGE and immunoblotted with antibodies against FLAG and BLK.

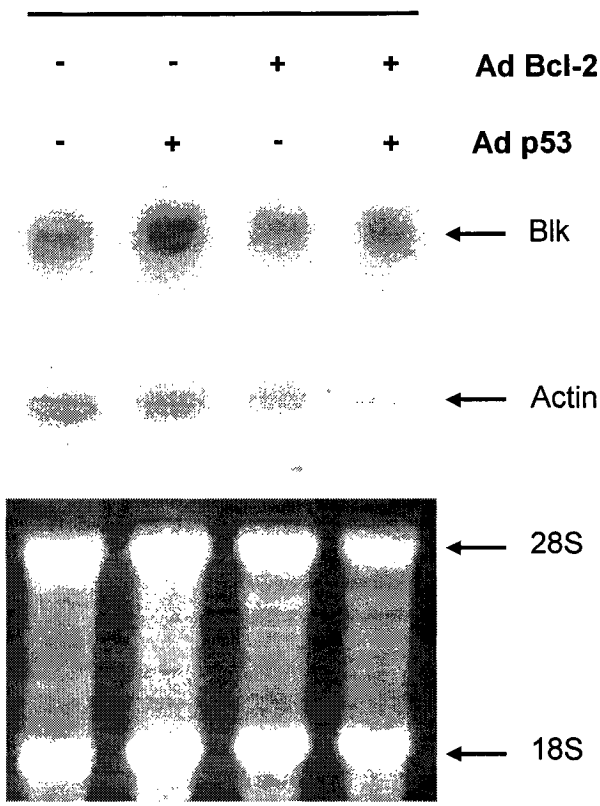

Figure 4 – *p53 does not induce BLK RNA.* Murine Renca cells were infected with Ad p53 vector in the presence or absence of Ad Bcl-2 vector. 24 h later, RNA was extracted from cells and 25 μg separated by agarose gel electrophoresis, transferred to nylon membrane, hybridized with α-^{32}P-labeled BLK cDNA, and the membrane exposed to radioautography. The membrane was stripped and probed with a α-^{32}P-labeled ACTIN cDNA. Shown in the bottom panel is a photograph of the ethidium bromide stained agarose gel prior to membrane transfer. The bands corresponding to 28S and 18S ribosomal RNA are indicated and provide gelloading controls.

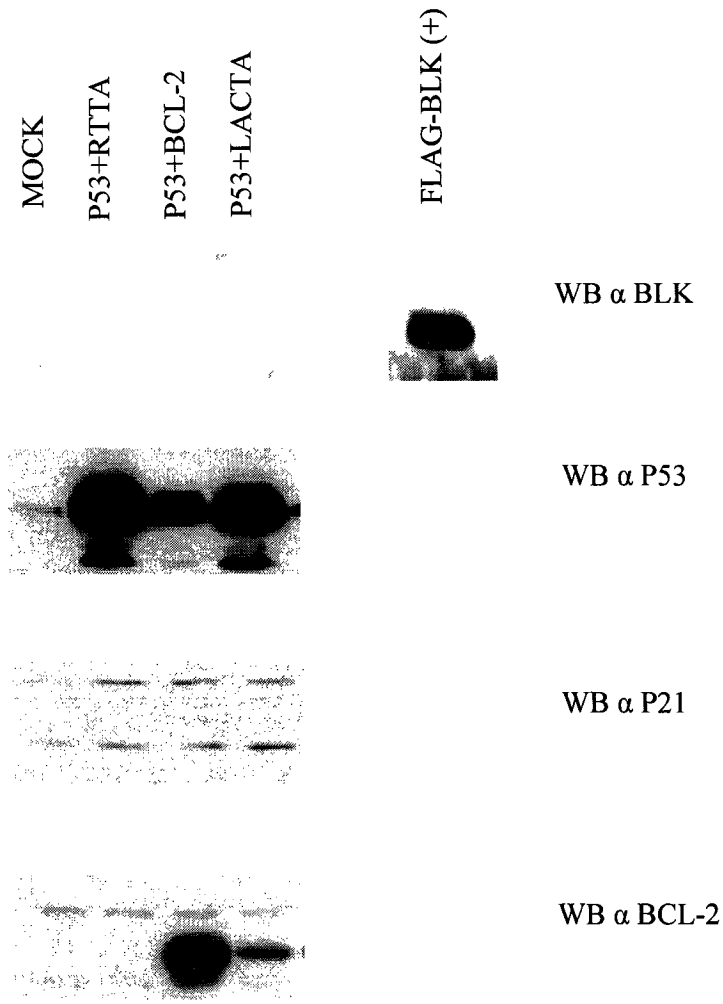

Figure 5 – *p53 does not induce BLK protein.* Murine Renca cells were infected with Ad p53 vector in the presence of Ad rTTA vector, Ad Bcl-2, or 10 μM lactacystin(LACTA). After 24 h, cell extracts were prepared and aliquots containing equivalent amounts of proteins were resolved by SDS-PAGE and immunoblotted with antibodies against BLK, P53, P21, and BCL-2. The upper right panel is a sample from BLK-expressing cells(control).

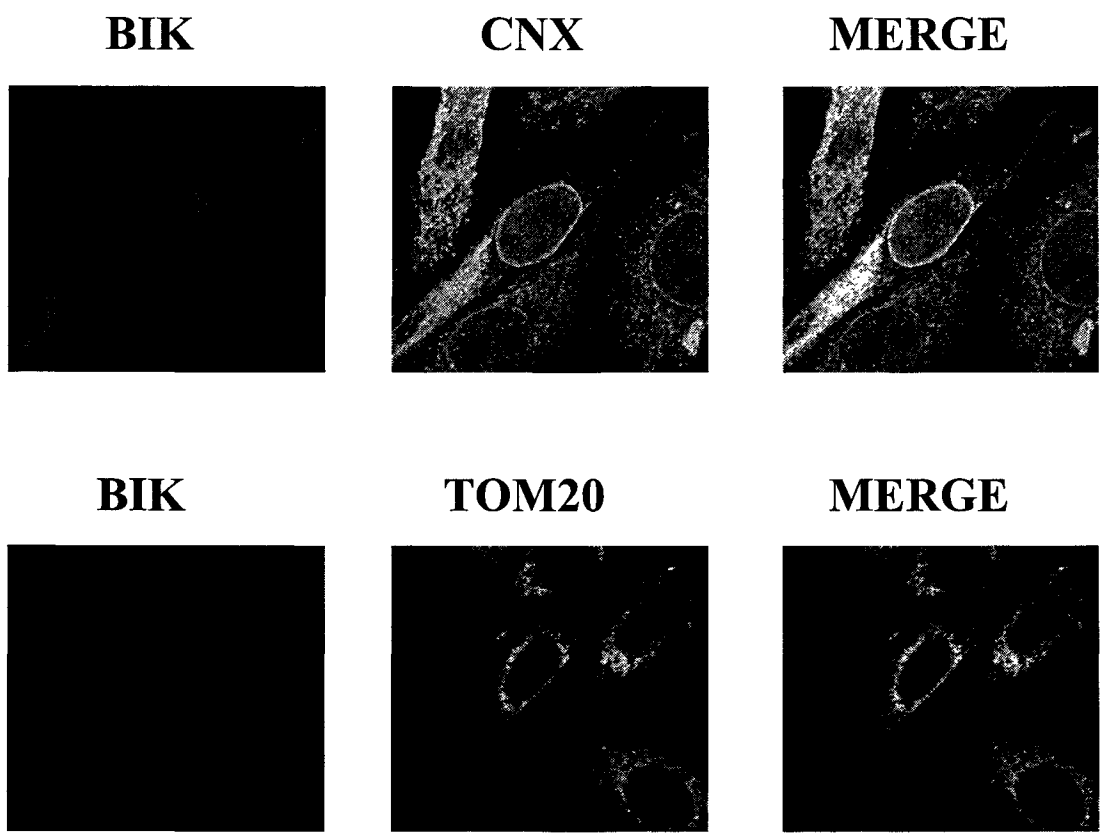

Figure 6A – Subcellular distribution of BIK. (a) 3T3 cells grown on coverslips were transfected with plasmid expressing FLAG-BIK. The cells were double-stained with anti-FLAG (Alexa 594 (red)) and either anti-CNX(calnexin, ER marker) or anti-TOM20(mitochondrial outer membrane marker)(Alexa 488 (green)), and images of the same cell visualized in the red, green, or merged(yellow) channels.

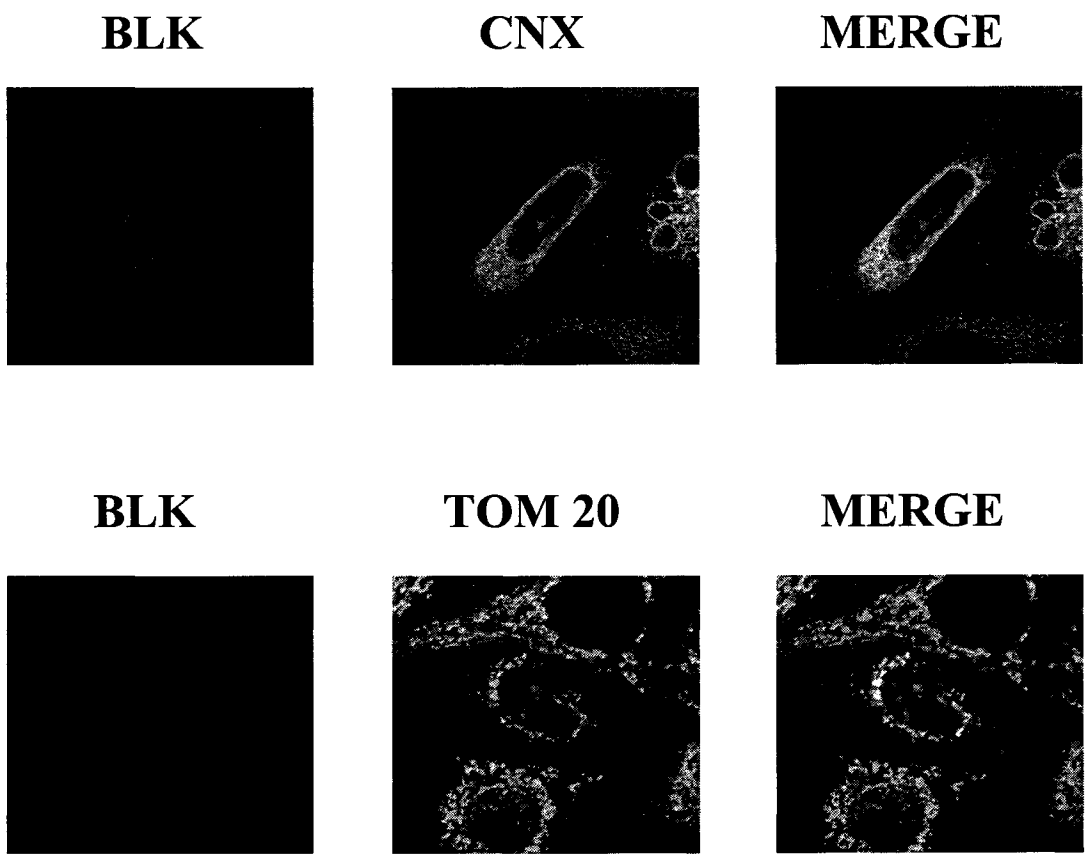

Figure 6B – Subcellular distribution of BLK. (a) 3T3 cells grown on coverslips were transfected with plasmid expressing FLAG-BLK. The cells were double-stained with anti-FLAG (Alexa 594 (red)) and either anti-CNX(calnexin, ER marker) or anti-TOM20(mitochondrial outer membrane marker)(Alexa 488 (green)), and images of the same cell visualized in the red, green, or merged(yellow) channels.

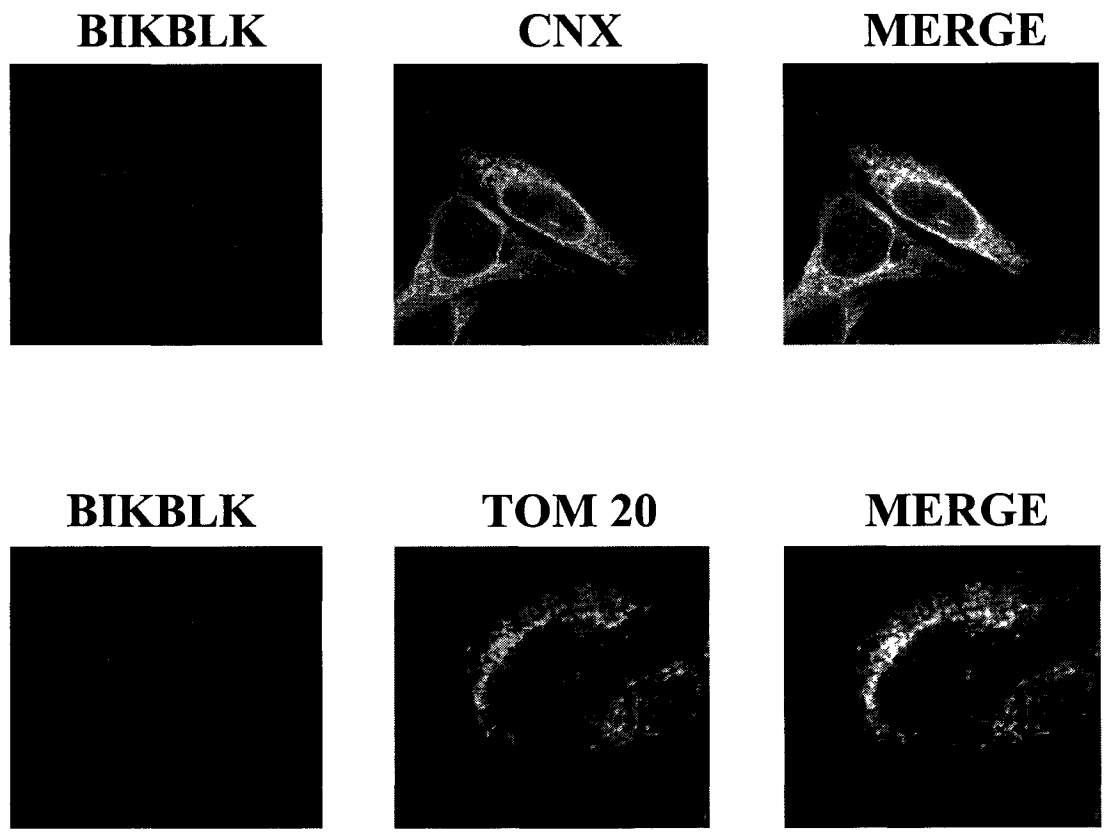

Figure 6C – Subcellular distribution of BIKBLK. (a) 3T3 cells grown on coverslips were transfected with plasmid expressing FLAG-BLK. The cells were double-stained with anti-FLAG (Alexa 594 (red)) and either anti-CNX(calnexin, ER marker) or anti-TOM20(mitochondrial outer membrane marker)(Alexa 488 (green)), and images of the same cell visualized in the red, green, or merged(yellow) channels.

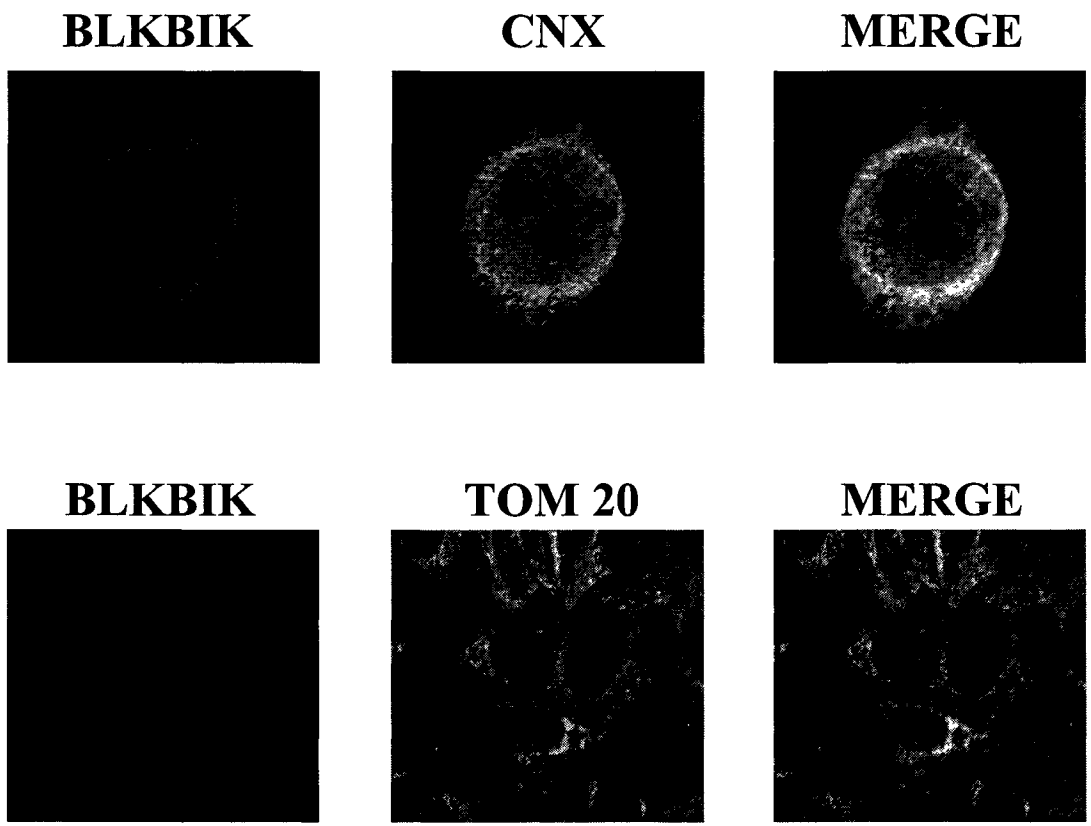

Figure 6D – Subcellular distribution of BLKBIK. (a) 3T3 cells grown on coverslips were transfected with plasmid expressing FLAG-BLK. The cells were double-stained with anti-FLAG (Alexa 594 (red)) and either anti-CNX(calnexin, ER marker) or anti-TOM20(mitochondrial outer membrane marker)(Alexa 488 (green)), and images of the same cell visualized in the red, green, or merged(yellow) channels.

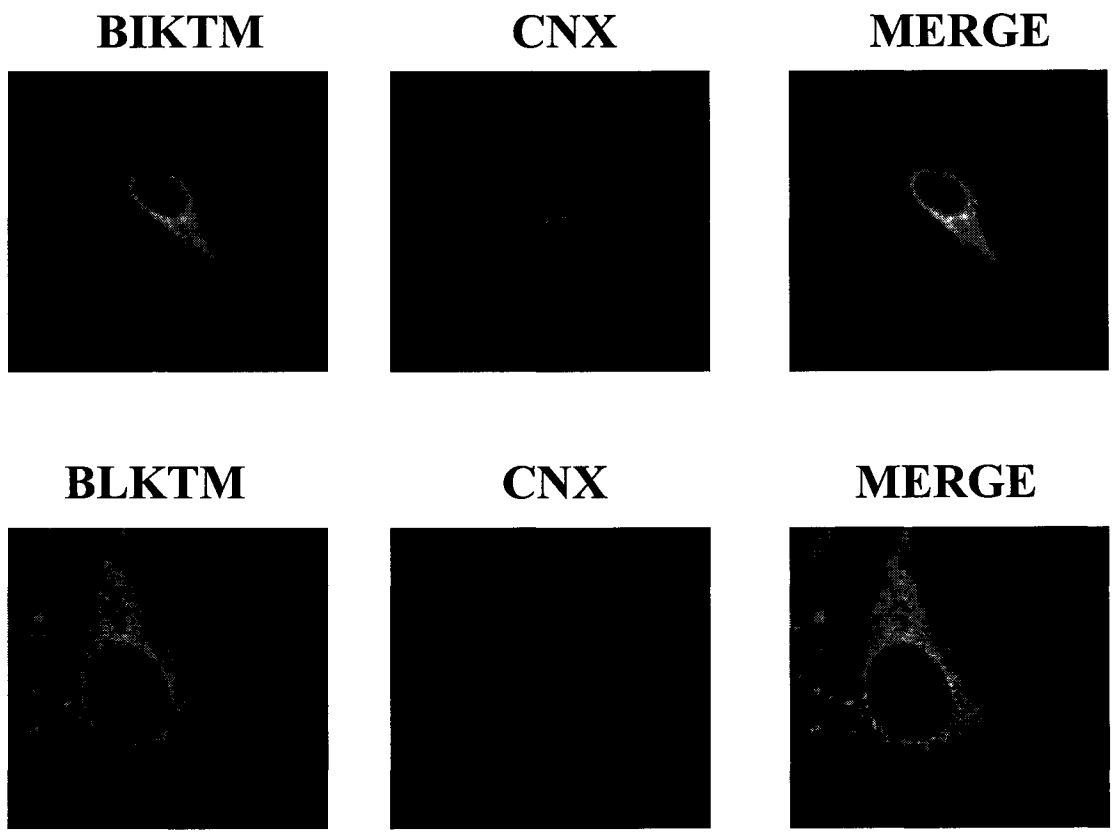

Figure 6E – Subcellular distribution of BIKTM and BLKTM. (a) 3T3 cells grown on coverslips were transfected with plasmid expressing EGFP-BLKTM or EGFP-BIKTM(green). The cells were stained with anti-CNX(Alexa 594 (red)) , and images of the same cell visualized in the red, green, or merged(yellow) channels.

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