BH3-only BIK Regulates BAX, BAK-dependent Release of Ca^{2+} from Endoplasmic Reticulum Stores and Mitochondrial Apoptosis during Stress-induced Cell Death*

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BIK, a pro-apoptotic BH3-only member of the BCL-2 family, targets the membrane of the endoplasmic reticulum (ER). It is induced in human cells in response to several stress stimuli, including genotoxic stress (radiation, doxorubicin) and overexpression of E1A or p53 but not by ER stress pathways resulting from protein misfolding. BIK initiates an early release of Ca^{2+} from ER upstream of the activation of effector caspases. Release of the mobile ER Ca^{2+} stores in baby mouse kidney cells doubly deficient in BAX and BAK, on the other hand, is resistant to BIK but is sensitive to ectopic BAK. Over-expression of p53 stimulates recruitment of BAK to the ER, and both its recruitment and assembly into higher order structures is inhibited by BIK small interfering RNA. Employing small interfering RNA knock-downs, we also demonstrated that release of ER Ca^{2+} and mitochondrial apoptosis in human epithelial cells requires BIK and that a Ca^{2+}-regulated target, the dynamin-related GTPase DRP1, is involved in p53-induced mitochondrial fission and release of cytochrome c to the cytosol. Endogenous cellular BIK, therefore, regulates a BAX, BAK-dependent ER pathway that contributes to mitochondrial apoptosis.

Utilizing a DNA microarray analysis of genes that are stimulated by the oncogenic E1A protein of adenovirus, we previously identified BH3-only BIK as a strong responder in human KB epithelial cells. E1A is a potent inducer of both BIK protein and apoptosis in human epithelial cells, dependent on its ability to up-regulate the levels of p53 (1). Moreover, overexpression of p53 in p53-null lung H1299 cells also induced BIK mRNA and protein with kinetics very similar to the induction of p21^{WAF1}, which is a rapid response protein in this pathway. Additionally, BIK is induced in estrogen-dependent MCF7 breast cancer cells in response to inhibition of estrogen signaling (2), and induction of BIK contributes to the apoptotic selection of mature B lymphocytes (3). The fact that BIK is primarily regulated through induction of BIK protein is consistent with studies indicating that BIK is a constitutively active pro-apoptotic protein.

The murine ortholog of BIK, Blk, is largely restricted to hematopoietic and endothelial cells and, in contrast to BIK, is not induced by genotoxic stress (4). Moreover gene deletion had little if any effect on the sensitivity of murine cells to genotoxic stress, and animals developed normally (4). In contrast to most BH3-only proteins in mouse and man, which exhibit a high degree of amino acid sequence identity (5, 6), the human and mouse orthologs of BIK are only 42.5% identical, despite having very similar gene structures (7, 8). Consistent with the findings reported by Coultas et al. (4), we also have found no evidence that Blk mRNA or protein is induced by either genotoxic stress or p53 overexpression in a variety of mouse cell lines and primary cell cultures. Remarkably, therefore, murine Blk and human BIK respond differently to stress stimuli. Consistent with the findings that human BIK may contribute to tumor suppression, there is reported evidence that mutation of the BIK gene is a frequent feature of B-cell lymphomas (9), and the chromatin locus 22q13.3, which contains BIK, exhibits deletions in human breast and colorectal cancers (10). To better understand the contribution of BIK induction to apoptosis in human epithelial cells, we utilized BIK RNA interference. BH3-only BIK interacts with the multi-BH domain anti-apoptotic members of the BCL-2 family but not with pro-apoptotic BAX and BAK (11–15). It contains a single transmembrane segment at its extreme COOH terminus, but in contrast to most BH3-only proteins, which target mitochondria, BIK is integrated almost exclusively in the membrane of the endoplasmic reticulum (ER) (2) (1, 14). Although other members of the BCL-2 family, including anti-apoptotic BCL-2 itself and the multidomain BAX and BAK pro-apoptotic effector molecules, also target the ER (reviewed in Ref. 33), the role of the ER in supporting the mitochondrial apoptosis pathway is only now beginning to emerge (15–17). In the Fas death pathway, for example, cleavage of BAP31 at the ER membrane 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 (18). DRP1 is responsible for scission of the outer membrane during normal mitochondrial fission and fusion, and in the absence of fusion, converts the tubular “worm-like” network of steady-state mitochondria into punctiform fragments (19, 20). Such DRP1-dependent mitochondrial fragmentation is an early event in several apoptotic pathways (21), and in these pathways, DRP1 appears to be necessary for effective egress of cytochrome c from the organelle to the cytosol (15, 21). Cytoplasmic cytochrome c, in

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1 J. P. Mathai, M. Germain, and G. C. Shore, unpublished data.
2 The abbreviations used are: ER, endoplasmic reticulum; siRNA, small interfering ribonucleic acid; HA, hemagglutinin; LM, light membrane; tTα, reverse tet transactivating protein; RNAi, RNA interference; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; DKO, double knock-out.
turn, becomes a critical constituent of the apoptosome, which processes and activates effector procaspases (22, 23).

Recent work has established that BAX, BAK regulates ER Ca\(^{2+}\) homeostasis (24, 25). Here we employed an adenovirus containing p53 as a tool to induce endogenous BIK in p53-null H1299 human lung epithelial cells. Utilizing BIK siRNAs, we demonstrated that BIK induction in this system is required to initiate early release of Ca\(^{2+}\) from the ER, mitochondrial fragmentation, and activation of the mitochondrial cytochrome c release pathway. BIK initiates the release of Ca\(^{2+}\) from ER stores by a pathway dependent on BAX, BAK.

**EXPERIMENTAL PROCEDURES**

**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 (26), were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin and penicillin. Transformed baby mouse kidney epithelial and H1299 lung carcinoma cell lines, either expressing or not expressing HA-BCL-2, were infected with either 2 μg thapsigargin or infected with Ad p53 for the indicated times. Gy, gray; wt, wild type; BIP, binding protein.

**RNA Interference of BIK and Viral Infection**—Thapsigargin-releasable ER calcium 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 (15, 29). In brief, 2 × 10^6 cells were harvested 24 h later and lysates assessed for luciferase activity using a Lumat LB 9507 luminometer and the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instruction. Cu\(^{2+}\) Measurements—Thapsigargin-releasable ER calcium was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Cu\(^{2+}\)-free buffer (15, 29).

**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. The cells well harvested 24 h later and lysates assessed for luciferase activity using a Lumat LB 9507 luminometer and the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instruction.

**NOXA Cloning and Northern Blots**—NOXA cDNA was cloned as described in Ref. 1 using the sequences deposited in GenBank™ (accession number D90070). The primers used for the cloning of NOXA cDNA were forward 5'-TTGGATCCCTCCAGTTGGAGGCTGAGGTT-3' and 5'-CGGAATTCCTTGAAGGAGTCCCCTCATGC-3'. NOXA cDNA was cloned as described in Ref. 1 using 30 μg of total RNA extracted from H1299 cells or KB cells, either expressing or not expressing ectopic HA-BCL-2.

**RNA Interference of BIK and Viral Infection**—The following siRNA duplexes, with a 3'-end dT overhang and corresponding to two separate regions within the BIK RNA sequence, were purchased from Drmarcon Research (Lafayette, CO) (numbers are in relation to the start site nucleotide for translation): siRNA BIK145, 5'-AUGCAUGGAGGGCAGUGAC3'; siRNA BIK315, 5'-GUUUAUGGAGGAGGCUU-3'. Double-stranded siRNA duplex 5'-CUUACCGUGAGAUCUGA-3' with a 3'-end dT 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/transfection was 60 nM. Adenoviral infection of cells was performed ~12 h after transfection with siRNA as described previously (27), using 100 plaque-forming units/cell of virus.

**Antibodies, Immunoblots, Immunofluorescence, and Microscopy**—The following antibodies were utilized: goat anti-BIK (Santa Cruz Biotechnology), mouse anti-actin (ICN Biomedicals), rabbit anti-TOM20 (described in Ref. 42), monoclonal anti-p53 (Pharmingen), rabbit anticalnexin and rabbit anti-binding protein (gift from J. Bergeron), mouse anti-cytochrome c (Pharmingen), rabbit anti-BAX (Santa Cruz Biotechnology), 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 (1, 15). For immunofluorescence, cells were plated onto coverslips at ~50% confluency for transfection and adenoviral infection. After the indicated infection times, the cells were treated and visualized as previously described (15). In experiments for Fig. 5C, all cells were treated with 5 μM nocodazole for 20 min prior to PFA fixing to aid in the visualization of fission events (26).

**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. The cells were well harvested 24 h later and lysates assessed for luciferase activity using a Lumat LB 9507 luminometer and the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instruction. Cu\(^{2+}\) Measurements—Thapsigargin-releasable ER calcium was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Cu\(^{2+}\)-free buffer (15, 29). In brief, 2 × 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% bovine serum albumin, 250 mM sodium pyruvate). The cells were resuspended in 200 μl of calcium-free buffer containing 0.5% pluronic acid and subsequently loaded with the cell-permeable fluorescent indicator Fura-2/AM at 3 mM for 30 min at 37 C. After a final wash, the cells were resuspended in Cu\(^{2+}\)-free buffer and a 340/380-nm excitation ratio at a 510-nm emission wavelength were obtained using a LS 50B PerkinElmer Life Sciences luminescence spectrophotometer. For Fig. 6, the cells were grown and treated on poly-L-lysine-treated coverslips and loaded by adding 100 μl Fura-2/AM to the culture medium for 30 min. Coverslips were washed with Hanks' buffer (3 mM Na\(_2\)HPO\(_4\), 5.4 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM HEPES, 10 mM glucose, 137 mM NaCl, 4.2 mM MgCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM HEPES, 10 mM glucose, 137 mM NaCl, 4.2 mM MgCl\(_2\)), followed by two washes with Ca\(^{2+}\)-free Hanks' Buffer (3 mM Na\(_2\)HPO\(_4\), 5.4 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM HEPES, 10 mM glucose, 137 mM NaCl, 4.2 mM MgCl\(_2\)). Images were obtained as previously described (30) using an intensified charge-coupled device camera (IC2000) and PTI (Photon Technology International Inc., Princeton, NJ) software at a single emission wavelength (510 nm) with a double excitation wavelength (540 and 580 nm).
380 nm). Fluorescence ratio (340/380) was measured in cells treated with 2 μM thapsigargin and the Fura-2 ratio values converted to \([\text{Ca}^{2+}]\) according to the formula of Grynkiewicz et al. (31). The peak thapsigargin-releasable \([\text{Ca}^{2+}]\)cyto was calculated as the difference in cytosolic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca²⁺-free Hanks' buffer.

RESULTS AND DISCUSSION

BIK Expression Is Induced by Oncogenic and Genotoxic but Not ER Stress—We have previously shown that BIK is induced by adenovirus E1A in a p53-dependent manner. The resulting

Fig. 2. siRNA-BIK315 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 h 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 h and luciferase activity measured. Shown are mean ± S.D. of three independent experiments. C, H1299 cells were transfected with the indicated siRNAs and infected with either Ad p53 or control Ad rtTa. The cell lysates were collected and analyzed for BIK expression by SDS-PAGE and immunoblotting using the indicated antibodies. LUC, luciferase.

380 nm). Fluorescence ratio (340/380) was measured in cells treated with 2 μM thapsigargin and the Fura-2 ratio values converted to \([\text{Ca}^{2+}]\) according to the formula of Grynkiewicz et al. (31). The peak thapsigargin-releasable \([\text{Ca}^{2+}]\)cyto was calculated as the difference in cytosolic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca²⁺-free Hanks' buffer.

Fig. 3. 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 h. Cells were visualized by phase contrast light microscopy. C, the detached cells from the culture medium 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 five independent experiments. D, as in B, caspase-3 like protease activity was measured by the ability of cell lysates to hydrolyze the fluorogenic caspase substrate DEVD-7-amino-4-methylcoumarin. Data presented are means ± S.D. for three independent experiments and are expressed as the fold increase in DEVDase activity compared with 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). LUC, luciferase; RFU, relative fluorescence units.

BIK protein accumulates to especially high levels in cells expressing BCL-2, because BIK is induced upstream of BCL-2 and does not decay in these BCL-2-protected cells (1). In Fig.
expression of BIK mRNA, together with that of another p53-inducible gene product, NOXA, was assessed by Northern blots of total RNA following infection of KB epithelial cells (p53 wild type) with Ad E1A, which elicits a strong pro-apoptotic stress stimulus. The adenoviral vector Ad5 dl520E1B was used for this purpose (32), which delivers only the pro-apoptotic 243-amino acid E1A12S oncoprotein, with no E1B products, which are protective against cell death agonists. Pro-apoptotic cell stress can also be initiated by overexpressing p53 itself in p53-null cells (1). 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 Ad E1A or Ad p53 (time 0, Fig. 1A). The subsequent increase of BIK mRNA in response to these inducers, however, was robust. In contrast to BIK protein levels (1), BCL-2 did not strongly influence BIK mRNA levels. Because BIK protein is induced by E1A in a p53-dependent manner (1), we also examined stimuli that up-regulate endogenous p53 in KB cells. As shown in Fig. 1B, genotoxic damage conferred by exposure of the cells to 25 gray of γ radiation or treatment with 0.4 μg/ml topoisomerase inhibitor doxorubicin also stimulated BIK protein induction in parallel with the accumulation of p53. Because BIK is strongly concentrated at the ER from where it is able to exert its pro-apoptotic function independent of a mitochondrial association (1, 14, 33), we also sought to determine whether BIK induction might occur in response to ER stress stimuli. To that end, we treated H1299 cells overexpressing BCL-2 with either Ad-BIK or Ad rtTa in the presence of 50 μM Z-VAD-fmk for 13 h. ER and mitochondrial fractions were isolated and treated as in A. HM, heavy membrane.

FIG. 4. p53-regulated ER calcium sensitivity is diminished by BIK knockdown. A, H1299 cells were infected with Ad BIK in the presence (open circle) or absence (closed square) of 50 μM Z-VAD-fmk, and cell viability was measured as the percentage of cells that excluded trypan blue at the indicated times. B, p53-induced ER calcium release is reduced by BIK knockdown. H1299 cells were transfected with siRNA-BIK315 or siRNA-LUC, followed by infection with either Ad p53, Ad BIK, or control Ad rtTa for 14 h. The cells were then loaded with Fura-2/AM, and peak cytosolic Ca2+ concentrations were measured as the difference in Fura-2 fluorescence recorded before and after the addition of 2 μM thapsigargin. Data is presented relative to that of untreated cells. Shown are the mean ± S.D. of five independent experiments. LUC, luciferase.

FIG. 5. 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 Z-VAD-fmk. Light membrane (LM) and mitochondrial fractions were isolated 13 h after infection, treated with 0.5 mM bismaleimidoethane (BMH) or Me2SO (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 Z-VAD-fmk for 13 h. ER and mitochondrial fractions were isolated and treated as in A. HM, heavy membrane.
ever, no evidence of BIK induction was observed despite the observed induction of binding protein by 24 h. Similar observations were made with another ER stressor, tunicamycin, and with different cell lines. Moreover, the times at which the presence of BIK was examined overlapped with the appearance of dying cells (data not shown). BIK regulation, therefore, is not sensitive to ER stress.

**Knockdown of BIK by siRNA**—To investigate the role of BIK in situations where it is induced, we employed RNA interference (RNAi). To this end, we designed the small interfering ribonucleic acid (siRNA) duplexes siRNA-BIK145 and siRNA-BIK315, which are homologous to regions within the BIK coding sequence initiating at nucleotides 145 and 315 relative to the start site of translation, respectively. An HA-BIK mutant harboring a disabling point mutation within its BH3 region (L61G), which permits high accumulation of the protein (1), was co-transfected with siRNAs BIK315 or BIK145 or a control siRNA targeting the luciferase gene within the pGL3-CMV vector (designated siRNA-LUC, Fig. 2A). siRNA-BIK145 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 siRNA-BIK145, the vector pRL-CMV, which encodes the gene for Renilla 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 h. As shown in Fig. 2B, the siRNA-LUC inhibited nearly all firefly luciferase activity, whereas siRNA-BIK145 had no effect on activity as compared with the control. Thus siRNA-BIK315 is both a strong and specific inhibitor of BIK protein expression. In Fig. 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 (Fig. 2C) more effectively than its ability to counter the large amount of BIK(L61G) generated in BIK-transfected cells (Fig. 2A) is consistent with siRNA-BIK145 exhibiting intermediate effectiveness against its target. Thus, siRNA-BIK315 serves as an effective means to specifically knockdown 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 Ad p53**—Fig. 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 h 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 (1) (Fig. 3B). Transfection with siRNA-BIK315 inhibited 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 control adenovirus vector encoding reverse tet transactivating protein (Ad rtTa) (Fig. 3B), with over three times the number of cells remaining adherent to cell culture plates compared with that of the siRNA-LUC control (Fig. 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 knock down by siRNA-BIK315 of both endogenous BIK induced by Ad p53 and ectopic BIK expressed by Ad BIK (Fig. 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 (Fig. 3D, gel insert), substantial effector caspase activity was still observed, although 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 siRNA315 (Fig. 3D, gel insert), where 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 knockdown, which further validates the specificity of the BIK siRNA and confirms that BIK plays an important role in the stress-induced apoptosis elicited by over-expression of p53.

**BIK Mediates Early Ca2+ Release from ER**—Emerging evidence suggests that Ca2+ signaling by the ER contributes to the mitochondrial apoptosis pathway (15, 21). Because these ER-mediated events occur upstream of activation of effector caspases (15), we focused our analysis at earlier times (14 h post-infection) following infection of cells with Ad p53. Moreover, we included 50 μM Z-VAD-fmk in all subsequent assays, because this inhibitor effectively blocks the activation of caspases (11, 20) and loss of cell viability (Fig. 4A) that can result from exposure of cells to BIK over an extended time period.

Consistent with a role for BIK in this ER calcium signaling, we found that infection of H1299 cells with Ad BIK in the presence of Z-VAD-fmk induced early and robust release of Ca2+ from ER stores, whereas the control adenovirus vector, Ad rtTa, did not (Fig. 4B, right). The loss of ER Ca2+ was measured by loading cells with the cytosolic Ca2+-sensitive dye Fura-2 in the absence of extracellular Ca2+ and determining the difference in peak [Ca2+]cyto before and after the addition of thapsigargin, which causes immediate depletion of ER calcium stores. Similar to Ad BIK, Ad p53 also induced an early loss of ER Ca2+ (14 h post-infection) to an extent similar to that seen for Ad BIK, and importantly, this response to Ad p53 was
BIK triggers recruitment and oligomerization of BAK at the ER—Recent evidence has indicated that, in addition to targeting mitochondria, a relatively small fraction of total cellular BAX and BAK can also reside at the ER, where they undergo oligomerization in response to stress stimuli (25, 34, 35). It has been further suggested that BAK and BAX regulate ER Ca²⁺ stores and, through this mechanism, influence multiple apoptotic signals (25). Because oligomerization of BAK and BAX typically involves BH3-only proteins, we investigated whether BIK might influence 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 h in the presence of 50 μM Z-VAD-fmk. Light membranes (LM, enriched in ER membranes) and heavy membranes (enriched in mitochondria) were collected and incubated with the sulfhydryl-reactive chemical cross-linking agent bismaleimidoethoxane 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 (Fig. 5A). Moreover, bismaleimidoethoxane treatment of LM resulted in the appearance of higher order BAK oligomers following p53 expression (Fig. 5A). The LM was not contaminated with mitochondria, as indicated by the absence of the mitochondrial outer membrane-resident protein TOM 20. In Fig. 5, the exposure time of the blots was selected to optimize BAK resolution; in fact, the amount of BAK that distributes to the LM following p53 stimulation is small (10–15%) relative to the pool that is in the heavy membrane fraction. Of note, the ability of Ad p53 to induce localization and oligomerization of BAK at the ER were retarded by siRNA-BIK315 compared with control siRNA-LUC (Fig. 5A, lanes 1 and 2). To examine the ability of BIK on its own to initiate these events, control H1299 cells or H1299 cells stably overexpressing BCL-2 (1) were infected for 12 h with an Ad BIK or control Ad rtTa, in the presence of 50 μM Z-VAD-fmk. Fig. 5B shows that, similar to Ad p53, Ad BIK was also able to trigger BAK ER recruitment and oligomerization. As expected for a BH3-only protein, these BIK-induced events were inhibited by the overexpression of BCL-2.

Effects of BAX, BAK Gene Deletion—The ability of Ad BIK to release mobile stores of Ca²⁺ from the ER was then examined in transformed baby kidney epithelial cells derived from BAX, BAK doubly deficient (DKO) mice (36). As previously documented for embryonic fibroblast cells (25), [Ca²⁺]ER is somewhat lower in DKO epithelial cells compared with wild type (Fig. A). Of note, however, strong release of ER Ca²⁺ was observed upon overexpression of ectopic BAK in these DKO cells in the presence of 50 μM Z-VAD-fmk (Fig. 6B), indicating that, as in wild-type cells, these ER stores of Ca²⁺ can indeed be mobilized in response to

**Fig. 7.** BIK knockdown inhibits p53-induced fission of mitochondria, cytochrome c release, and BAX/BAK activation. A. BIK knockdown mitigates p53-induced mitochondrial fission. H1299 cells were transfected with either siRNA-BIK315 or siRNA-LUC, followed by infection with Ad p53 or control Ad rtTa for 14 h in the presence of 50 μM Z-VAD-fmk. The cells were then fixed and stained with anti-TOM 20 antibody (representative images are shown). B, the percentage of cells from A with mitochondrial fission was scored. Shown is the mean ± S.D. of four independent experiments. C, 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 Z-VAD-fmk. 13 h 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 ± S.D. of three independent experiments. D, BIK knockdown diminishes p53-induced cytochrome c (Cyt. c) release and BAX/BAK activation. Transfection was done as in A, except the coverslips were fixed at 16 h after infection with Ad p53 or control Ad rtTa 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. E, the cells in D were scored for BAX and BAK activation, as well as cytochrome c release. Shown is the mean ± S.D. of three independent experiments.
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Changes were strongly inhibited by siRNA-BIK315 (Fig. 7 bottom panel). Mitochondria in this pathway. Moreover, BIK knockdown inhibited the ER from where it elicits pro-apoptotic signals and, given the association with BIK expression, therefore, we focused on the initiating events that are strongly inhibited by the wide-spectrum caspase inhibitor Z-VAD-fmk (1, 14). To investigate the initiating events associated with BIK expression, therefore, we focused on the initiating events that are strongly inhibited by the wide-spectrum caspase inhibitor Z-VAD-fmk (1, 14). To investigate the initiating events associated with BIK expression, therefore, we focused on the initiating events that are strongly inhibited by the wide-spectrum caspase inhibitor Z-VAD-fmk (1, 14).

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REFERENCES

1. Mathai, J. P., Germain, M., Marcellus, R. C., and Shore, G. C. (2002) Oncogene 21, 2534–2544
2. Kojima, J., Chneses, J., Cosen, K. R., Lee, R. S., Gekk, P., Isselbacher, K. J., and Shiolda, T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2351–2356
3. Jiang, A., and Clark, E. A. (2001) J. Immunol. 166, 6025–6033
4. Coullas, L., Bouillet, F., Stanley, E. G., Brodnecki, T. C., Adams, J. M., and Strasser, A. (2004) Mol. Biol. Cell 15, 1570–1581
5. Bouillet, P., and Strasser, A. (2002) J. Cell Sci. 115, 1567–1574
6. Puthalakath, H., and Strasser, A. (2002) Cell Death Differ. 9, 505–512
7. Vena, S., Budar, M. L., Emmanuel, B. S., and Chinnadurai, G. (2000) Gene (Amst.) 254, 157–162
8. Amanna, I. J., Clise-Dwyer, K., Nashold, F. E., Hoag, K. A., and Hayes, C. E. (2001) J. Immunol. 167, 6069–6072
9. Barnhart, B. C., Alappat, E. C., and Peter, M. E. (2002) Semin. Immunol. 15, 185–193
10. Castells, A., Gussella, J. F., Ramesh, V., and Rustgi, A. K. (2000) Cancer Res. 60, 2830–2839
11. Han, J., Sabattini, P., and White, E. (1996) Mol. Cell. Biol. 16, 5857–5864
12. Elangovan, B., and Chinnadurai, G. (1997) J. Biol. Chem. 272, 24494–24498
13. Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. H., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Latz, R. J., and Chinnadurai, G. (1995) Oncogene 11, 1921–1928
14. Germain, M., Mathai, J. P., and Shore, G. C. (2002) J. Biol. Chem. 277, 14053–14060
15. Breckenridge, D. G., Germain, M., Mathai, J. P., Nguyen, M., and Shore, G. C. (2005) Oncogene 24, 8608–8618
16. Thonemius, M. J., and Donald, C. W. (2005) J. Cell. Sci. 118, 4493–4499
17. Oakes, S. A., Opferman, J. T., Pozzan, T., Korsmeyer, S. J., and Scorrano, L. (2003) Biochim. Pharmacol. 66, 1335–1340
18. Breckenridge, D. G., Stojeanovic, M., Marcellus, R. C., and Shore, G. C. (2003) J. Cell. Biol. 160, 1115–1127
19. Smirnova, E., Shurland, D. L., Ryazantsev, S. N., and van der Bliek, A. M. (1999) J. Cell Biol. 143, 351–358
20. Laferriere, A. M., Zappaterra, M. D., Rube, D. A., and van der Bliek, A. M. (1999) Mol. Cell 4, 815–826
21. Frank, S., Gaume, B., Bergmann-Leitner, E. S., Leitner, W. W., Robert, E. G., Catez, F., Smith, C. L., and Youle, R. J. (2001) Dev. Cell 1, 515–525
22. Dextere, A., Hoyer, M., and Yuan, J. (2003) Oncogene 22, 8545–8557
23. Boström, K. M., and Salvesen, G. S. (2000) Biochem. Soc. Symp. 233–242
24. Oakes, S. A., Scorrano, L., Opferman, J. T., Bassik, M. C., Nishino, M., Pozzan, T., and Korsmeyer, S. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 105–110
25. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) Science 300, 135–139
26. Nguyen, M., Branton, E. F., Walton, P. A., Ottavi, Z. N., Korsmeyer, S. J., and Scorrano, L. (2003) J. Cell. Biol. 162, 16251–16254
27. Ng, F. W., Nguyen, M., Kwan, T., Branton, E. F., Nichols, D. W., Cromlish, J. A., and Shore, G. C. (1997) J. Cell. Biol. 139, 327–338
28. Smirnova, E., Griparic, L., Shurland, D. L., and van der Bliek, A. M. (2001) Mol. Biol. Cell 12, 2245–2256
29. Zuppini, A., Groenendyk, J., Cormack, L. A., Shore, G., Ogas, M., Bleackley, R. A., and Michalak, M. A. (2002) Biochemistry 41, 2850–2858
30. Tolloczko, B., Jia, Y. L., and Martin, J. G. (1995) J. Cell Biol. 133, 242–254
31. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
32. Shepherd, S. E., Howe, J. A., Mymryk, J. S., and Bayley, S. T. (1993) J. Virol. 67, 2944–2949
33. Germain, M., and Shore, G. C. (2005) Science’s STKE http://stke.sciencemag.org/cgi/content/full/stke/200503/55/e10
34. Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. (2003) J. Cell. Biol. 162, 59–69
35. Nutt, L. K., Papa, M., Pahler, J., Fanger, B. R., Roth, J., McConkey, D. J., and Swisher, S. G. (2002) J. Biol. Chem. 277, 9219–9225
36. Degenhardt, K., Chen, G., Lindsten, T., and White, E. (2002) Cancer Cell 2, 193–203

BAX DKO mice, we showed that both cell types maintain a mobile pool of ER Ca2+, whereas wild-type cells were responsive (Fig. 6B). Release of ER Ca2+ in response to BIK, therefore, is dependent on the pro-apoptotic BAX DKO setpoint.
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37. Pitts, K. R., Yoon, Y., Krueger, E. W., and McNiven, M. A. (1999) Mol. Biol. Cell 10, 4403–4417
38. Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L., and Youle, R. J. (2002) J. Cell Biol. 159, 931–938
39. Wang, B., Nguyen, M., Breckenridge, D. G., Stojanovic, M., Clemens, P. A., Kuppin, S., and Shore, G. C. (2003) J. Biol. Chem. 278, 14461–14468
40. Desagher, S., Osen-Sand, A., Nichols, A., Eekes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) J. Cell Biol. 144, 891–901
41. Griffiths, G. J., Dubrez, L., Morgan, C. P., Jones, N. A., Whitehouse, J., Corfe, B. M., Dive, C., and Hickman, J. A. (1999) J. Cell Biol. 144, 903–914
42. Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998) J. Cell Biol. 143, 207–215