BH-3-only BIK Functions at the Endoplasmic Reticulum to Stimulate Cytochrome c Release from Mitochondria*

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Stimulation of apoptosis by p53 is accompanied by induction of the BH-3-only proapoptotic member of the BCL-2 family, BIK, and ectopic expression of BIK in p53-null cells caused the release of cytochrome c from mitochondria and activation of caspases, dependent on a functional BH-3 domain. A significant fraction of BIK, which contains a predicted transmembrane segment at its COOH terminus, was found inserted in the endoplasmic reticulum (ER) membrane, with the bulk of the protein facing the cytosol. Restriction of BIK to this membrane by replacing its transmembrane segment with the ER-selective membrane anchor of cytochrome b5 also retained the cytochrome c release and cell death-inducing activity of BIK. Whereas induction of cell death by BIK was strongly inhibited by the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, the inhibitor was without effect on the ability of BIK to stimulate egress of cytochrome c from mitochondria. This benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone-insensitive pathway for stimulating cytochrome c release from mitochondria by ER BIK was successfully reconstituted in vitro and identified the requirement for components present in the light membrane (ER) and cytosol as necessary for this activity. Collectively, the results identify BIK as an initiator of cytochrome c release from mitochondria operating from a location at the ER.

Apoptosis is a highly regulated mechanism of cell death that is required for normal development and maintenance of tissue homeostasis in multicellular organisms. One of the key events in many types of apoptosis is the release of mitochondrial cytochrome c to the cytosol, along with other proapoptotic factors like Smac/Diablo and AIF (1, 2). Cytochrome c, in the presence of dATP/ATP, then triggers the formation of a complex containing procaspase-9 and APAF-1, which leads to activation of caspase-9. Caspase-9 is an initiator caspase that processes effector caspases, resulting in a cascade of proteolytic events and apoptotic death (3).

Diverse upstream death signals appear to be coupled to downstream transformations in mitochondria through the activation of members of a subgroup of the BCL-2 family of proteins, which contain only one of the four domains that define BCL-2 proteins, the BH-3 domain (4). One or more of these BH-3-only proteins, including BID, BAD, BIM, Bmf, and others (5, 6), become activated in response to a death signal, which typically causes their translocation to mitochondria. The resulting organelle dysfunction and cytochrome c egress depend on a second proapoptotic subgroup of the BCL-2 family located in the mitochondrial outer membrane, the effector molecules BAX and BAK (7, 8). The third subgroup of the BCL-2 family is antiapoptotic and, in addition to the BH-1, -2, and -3 domains found in the proapoptotic effectors BAX and BAK, contains a BH-4 domain. When present in the mitochondrial outer membrane in excess, antiapoptotic BCL-2 members, such as BCL-2 and BCL-XL, maintain organelle integrity even in the face of sustained death signaling (5, 9). Of note, however, these antiapoptotic BCL-2 proteins are also found in association with endoplasmic reticulum (ER) and nuclear envelope (10).

Surveillance of genome integrity is tightly coupled to regulation of apoptosis, primarily through the activity of the p53 tumor suppressor protein. p53 is a transcription factor activated by DNA damage and the expression of certain oncoproteins, resulting in either cell cycle arrest or apoptosis (11). Whereas its cell cycle arrest function is well defined, the molecular basis for proapoptotic signaling by p53 is only now emerging. It appears to operate by inducing the production of a number of constitutively active proapoptotic proteins, each of which is able to independently trigger apoptosis (12, 13). Two of these, Noxa (14) and Puma (15, 16), have been identified as death-inducing BH-3-containing proteins that target and breach mitochondrial integrity. Moreover, the APAF-1-caspase-9 complex has been shown to be necessary for p53 to induce apoptosis, thus implicating cytochrome c release as an important step in the p53 apoptotic pathway (17).

The BH3-only BCL-2 homologue BIK (18, 19) is up-regulated in p53-null H1299 cells infected with an adenovirus vector coding for wild-type p53. A significant fraction of cellular BIK localizes to ER membranes, where it can induce cytochrome c release from mitochondria. Both in vitro reconstitution experiments and engineered targeting of BIK to ER in vivo, using the heterologous cytochrome b5 transmembrane domain, revealed that this process is independent of a direct interaction between BIK protein and mitochondria and does not depend on BAX translocation/insertion in mitochondria. Collectively, our results suggest that BH3-only BIK is capable of initiating cytochrome c release from mitochondria and apoptosis from a location at the ER.

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1 The abbreviations used are: ER, endoplasmic reticulum; Ad, adenoviral vector; BKA, bongkrekic acid; HA, hemagglutinin; HM, heavy membrane; LM, light membrane; PARP, poly(ADP-ribose) polymerase; PTP, permeability transition pore; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.
FIG. 1. Induction of apoptosis by p53 in p53-null H1299 cells. A, time course of cell death. Cells were infected with a control adenovirus vector (Ad LacZ) (●) or Ad p53 in the presence (○) or absence (□) of 50 μM zVAD-fmk, and cell viability was measured as the percentage of cells ± S.D. that excluded trypan blue. Inset, at the indicated times, aliquots of whole cell lysates containing equivalent protein were subjected to SDS-PAGE and immunoblotted with antibody against p53. B, cytochrome c release from mitochondria and caspase activation following p53 induction. Cells were treated with Ad p53 for 20 h as indicated, fractionated into HM (enriched in mitochondria) and S100 (cytosol), and subjected to SDS-PAGE, and blots were probed with antibody against cytochrome c (Cyt c). For comparisons, the HM fractions were also probed with antibody against TOM20, and the cytosolic fractions were probed with antibody against γ-actin, which served as gel loading controls for the two fractions, respectively. Additionally, whole cell lysates were analyzed by immunoblotting with antibody against PARP, with the full-length protein (116 kDa) and 89-kDa apoptotic fragment indicated. C, insertion of BAX into mitochondrial membrane. Cells were treated and fractionated as in B. Mitochondria were analyzed by immunoblotting with antibodies against BAX and TOM20 either directly (−Alkali, lanes 1–4) or after extraction with 0.1 M Na2CO3, pH 11.5 (+Alkali, lanes 5–8). D, induction of BIK by p53 (as in A except that cell lysates were probed with antibodies against BIK and γ-actin (upper panel)); in the lower panel, relative levels of p53 and BIK were determined following infection of cells with Ad p53 by quantifying immunoblot signals using a Power Macintosh 7200/120 and NIH Image version 1.61 image analysis software. Representative results are presented.

EXPERIMENTAL PROCEDURES

Cell Culture and Infection with Adenovirus Vectors—H1299 lung carcinoma cells and KB epithelial cells were cultured in α-minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml streptomycin and penicillin. Cells were infected at 100 plaque-forming units/cell with adenovirus vectors expressing either wild-type p53, wild-type BIK tagged with hemagglutinin (HA) epitope at the N terminus, or a BH3 HA-BIK mutant in which leucine at position 61 was converted to glycine (L61G), as described (20, 21). Cells infected with Ad HA-BIK mutant in which the transmembrane domain (amino acids 135–160) has been replaced by the transmembrane domain of rabbit cytochrome b6 (amino acids 107–134) (FLAG-BIKb6TM). After 24 h, 1 × 10⁶ cells were centrifuged onto coverslips for 1 min at 2000 × g in a Cyto-Tek centrifuge (Sakura). The recovered cells were then fixed and analyzed by double label immunofluorescence. Cells were visualized with a Zeiss 510 confocal microscope, and images were captured and overlaid with the accompanying software.

Cell Fractionation—H1299 cells (50% confluence) were either mock-infected or infected with Ad vector for the indicated times, harvested, and suspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA) (approximately 10⁶ cells/ml). Cells were broken with 25 strokes in a motorized Teflon-glass homogenizer operating at 2000 rpm, and the homogenate was centrifuged at 1000 × g for 10 min to remove nuclei and cell debris (all subsequent steps were for 10 min). The supernatant was centrifuged at 9000 × g, the resulting pellet resuspended in 100 μl of HIM and recentrifuged at 9000 × g to give the heavy membrane (HM) fraction enriched in mitochondria. The supernatant from the first 9000 × g centrifugation, designated S9, was centrifuged at 170,000 × g to give the cytosolic supernatant (S100) and light membrane (LM) fractions. In certain instances, the HM and LM fractions were further processed by uniformly resuspending 10 μg of the membrane protein in 150 μl of 0.1 M Na2CO3, pH 11.5, and incubating on ice for 30 min. Alkaline-insoluble membrane protein was then recovered by centrifugation for 10 min at 170,000 × g. Protein concentrations were determined using the Bio-Rad protein assay.

In Vitro Cytochrome c Release Assay—S9 or S100 fractions (described above) were prepared from H1299 cells that were either mock-infected (control) or infected with Ad HA-BIK for 14 h in the presence of 50 μM zVAD-fmk and designated the “donor fractions.” HM, on the other hand, was prepared only from control H1299 cells (i.e., lacking BIK expression), resuspended at a concentration of 1 μg of protein/μl in CMRM (250 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K2HPO4), and designated the “acceptor fraction.” Alternatively, S100 and HM from mouse liver mitochondria were prepared as described (24). To measure cytochrome c release from mitochondria, S9 fractions containing 35 μg of protein in 12.5 μl of HIM or
its S100 and LM derivatives were mixed with 12.5 μl of cMBP containing HM (12.5 μg of protein) and 50 μl of zVAD-fmk and incubated at 37 °C for 30 min. The reaction mixture was then centrifuged at 13,000 × g for 10 min, and an aliquot of the supernatant was subjected to SDS-PAGE and immunoblotted with antibody against cytochrome c. For the assays using mouse liver mitochondria and suboptimal amounts of S9, 15 μg of S9 and 30 μg of mouse S100 were used. Input of mitochondria and recovery of the organelle in the pellet were typically monitored by blotting with antibody against the human mitochondrial protein import receptor located in the outer membrane, TOM20.

**RESULTS**

Delivery of an adenoviral vector expressing the wild-type p53 tumor suppressor protein (Ad p53) to the p53-null H1299 lung carcinoma cell line resulted in induction of p53 protein within 12 h, followed by cell death that was partially inhibited by the general caspase inhibitor zVAD-fmk (Fig. 1 A). Cytochrome c was released from mitochondria, and caspases were activated, as reflected by the cleavage of PARP (Fig. 1B). Although zVAD-fmk was able to completely block PARP cleavage, it did not have any effect on the release of cytochrome c, suggesting that the molecular events leading from p53 activation to cytochrome c release are not strongly dependent on zVAD-fmk-sensitive caspases. On the other hand, BCL-2 inhibited both cytochrome c release from mitochondria and PARP cleavage (Fig. 1B).

The proapoptotic BCL-2 homologue BAX is typically found loosely associated with HM enriched in mitochondria, in addition to its cytosolic localization in untreated cells. Upon apoptotic stimulus, however, it becomes integrated into the outer mitochondrial membrane and resistant to alkali extraction (25). As was seen for cytochrome c release, acquisition of alkali resistance of BAX in response to Ad p53 was insensitive to zVAD-fmk but was inhibited by BCL-2 (Fig. 1C).

In addition to the activation of BAX, p53 is believed to induce apoptosis by up-regulating multiple proapoptotic proteins, including members of the BH3-only class of the BCL-2 family,
such as Noxa (14) and Puma (15, 16). The BH-3-only protein BIK (18, 19) is another potential p53 target, since it was induced by the ectopic expression of p53 in H1299 cells, while being undetectable in control cells (Fig. 1D). Induction of BIK by Ad p53 occurred before the cells showed overt signs of loss of viability (starting at 24 h), as assessed by the exclusion of trypan blue (Fig. 1, A and D), with the kinetics of BIK induction paralleling that of cytochrome c release and the acquisition of BAX alkaline resistance (Fig. 1, B and C, and data not shown).

BIK Induces Cytochrome c Release from Mitochondria and Caspase Activation—To study directly BIK-induced apoptosis, an adenovirus expressing wild-type HA-tagged BIK was employed. Induction of BIK in p53-null H1299 cells using this system resulted in cell killing that was inhibited by zVAD-fmk (Fig. 2A), indicating the requirement for caspases. BIK also triggered the insertion of BAX into mitochondrial membrane (Fig. 2B). This was accompanied by loss of cytochrome c from mitochondria within 14 h of treatment and activation of caspases as judged by the cleavage of the caspase target BAP31 (23) (Fig. 2C). While zVAD-fmk prevented cell death (Fig. 2A) and caspase cleavage of BAP31 (Fig. 2C), it had little effect on membrane insertion of BAX and cytochrome c release from mitochondria, suggesting that, like p53, BIK-induced mitochondrial dysfunction is likely to be independent of zVAD-fmk-sensitive caspases. In addition, BIK activity required a functional BH3 domain, since a BIK mutant in which the conserved leucine 61 in the BH3 domain was mutated to a glycine (BIKL61G)) did not cause cell death (not shown), cytochrome c release, or caspase cleavage of BAP31 (Fig. 2C). Mutant BIK expression was higher than that of wild-type BIK (not shown).

A Significant Amount of BIK Localizes to the ER—In order to study the cellular localization of BIK, KB epithelial cells were transiently transfected with FLAG-tagged wild-type BIK and examined by immunofluorescence confocal microscopy. FLAG-BIK showed extensive co-localization with the ER marker calnexin, and this reticular network mostly comprised regions within the cell that did not include the mitochondrial outer membrane marker TOM20 (representative images are shown in Fig. 3A). Similarly, fractionation of H1299 cells infected with Ad HA-BIK revealed a co-distribution of HA-BIK and calnexin in the LM fraction. HA-BIK was also recovered in the HM fraction containing mitochondria, as judged by the presence of TOM20 (see Fig. 3B). Similar results were obtained with endogenous BIK following infection of H1299 cells with Ad p53 (data not shown). LM-associated BIK was resistant to alkali extraction but sensitive to proteinase K digestion (Fig. 3C), suggesting that BIK is integrated in the ER membrane and facing the cytosolic side. As controls, transmembrane calnexin was sensitive to proteinase K digestion (employing an antibody raised against its cytosolic tail), whereas the luminal chaperone BiP was resistant, and only BiP was extracted by alkali (Fig. 3C).

These experiments suggested that BIK might induce cytochrome c release and cell death from an ER location, although partial association of BIK with the mitochondria cannot be ruled out. Thus, to address the contribution of ER-localized BIK to cytochrome c release and cell death more fully, we generated a mutant BIK in which its C-terminal transmembrane segment of cytochrome b₅ (FLAG-BIK-b5TM), a sequence previously shown to selectively target fusion proteins to the ER (26, 27). The intracellular localization of this mutant was first studied by immunofluorescence confocal microscopy following transient transfection into KB cells. As shown in the representative images in Fig. 4A, FLAG-BIK-b5TM strongly co-localized with ER calnexin. Like FLAG-BIK, FLAG-BIK-b5TM was able to cause cytochrome c release from mitochondria in the presence of zVAD-fmk (Fig. 4B). Both proteins also induced cell death, as measured by a luciferase reporter essay (Fig. 4C) or by visual examination of cells co-transfected with vector expressing green fluorescent protein (not shown). That FLAG-BIK-b5TM triggered cytochrome c release from mitochondria and cell death argues that BIK can function through its location in the ER.

In Vitro Release of Cytochrome c from Mitochondria Lacking BIK by LM Containing HA-BIK—To establish that BIK lacking BIK by LM Containing HA-BIK—To establish that BIK is present in the ER can indeed stimulate cytochrome c release from mitochondria, the system was reconstituted in vitro (Fig. 5A). The assay comprised a donor and an acceptor fraction. The donor is an S9 extract from Ad HA-BIK-infected H1299 cells that contains LM and HA-BIK but that is free of mitochondria (as judged by the absence of TOM20) (Fig. 5B). The acceptor fraction is an HM fraction from uninfected cells that is enriched in mitochondria but contains no HA-BIK (Fig. 5B) or endogenous BIK (Fig. 1D). To minimize the influence of caspases, zVAD-fmk was both provided to the cells during Ad HA-BIK infection and included in the in vitro assays. The influence of the S9 or its derived S100 and LM components on recipient HM was determined by incubating donor fractions with acceptor HM in vitro for 30 min at 37 °C, recovering the HM by centrifugation at 13,000 × g, and measuring release of cytochrome c into the supernatant by immunoblotting. In all cases, equivalent amounts of S9 protein (35 μg) were added to the reactions.
Fig. 5. Donor S9 HA-BIK stimulates BAX membrane insertion and mitochondrial release of cytochrome c in an acceptor HM fraction lacking BIK.

A, the assay scheme. Donor S9 fraction was prepared from H1299 cells infected with Ad HA-BIK for 12 h in the presence of 50 μM zVAD-fmk. Centrifugation of S9 for 10 min at 170,000 × g yielded the S100 (supernatant (Sup.)) and LM (pellet) fractions. LM was resuspended in a volume of HIM buffer equal to that of S100. Acceptor HM (enriched in mitochondria) was prepared from mock-infected (CTRL) or Ad HA-BIK-infected cells and incubated at 37°C for 30 min with (+) or without (−) acceptor HM in the presence of 50 μM zVAD-fmk. At the end of the incubation, reaction mixtures were centrifuged at 13,000 × g to yield supernatants and pellets, which were probed with antibodies against cytochrome c (supernatant) and TOM20 (pellet). D, as in C except that donor fractions were prepared from cells infected with Ad vectors expressing either wild-type HA-BIK or mutant HA-BIK(L61G). E, donor S9 from Ad HA-BIK-infected cells was combined with acceptor HM and 10% removed and dissolved in SDS sample buffer (input). The remainder was incubated for 0 or 30 min at 37°C, and the HM was recovered. The resulting pellet and 10% input were immunoblotted with antibodies against HA and TOM20.

As shown in Fig. 5C, donor S9 from Ad HA-BIK-infected cells, but not from control cells, induced the release of cytochrome c from mitochondria. This effect was dependent on the presence of the LM, since their removal prevented cytochrome c release (Fig. 5C; compare S100 and S9). Cytochrome c release that was induced by LM-associated HA-BIK was also dependent on a functional BH3, since the L61G mutant was inactive (Fig. 5D). Of note, there was no detectable presence of HA-BIK recovered with the HM after incubation with donor HA-BIK S9 (Fig. 5E), whereas most of the TOM20 was recovered in this fraction. This indicates that stimulation of cytochrome c release from mitochondria by S9 HA-BIK did not occur because HA-BIK translocated from ER to mitochondria.

Efficient Cytochrome c Release by Ad HA-BIK in Vitro Is Blocked by Mitochondrial BCL-2 and Is Independent of PTP, Ca2⁺, and Mg2⁺—Although BIK can interact with antiapoptotic members of the BCL-2 family (18, 19), the in vitro reconstitution system afforded the opportunity to investigate the influence of BCL-2 operating downstream of BIK. This was addressed in the in vitro system by using HM from cells that overexpress BCL-2 (20). As shown in Fig. 6A, mitochondrial BCL-2 efficiently prevented cytochrome c release by HA-BIK S9. The influence of BCL-2 on the integrity of mitochondrial outer membrane and cytochrome c release has been suggested to be related to its effects on mitochondrial permeability transition pore (PTP) (28, 29). The involvement of PTP in the in vitro BIK-induced cytochrome c release was thus tested using the PTP inhibitor bongkrekic acid (BKA). As shown in Fig. 6B, 100 μM BKA did not block BIK-induced cytochrome c release, but it inhibited cytochrome c release by a known PTP activator, Ca2⁺, indicating that the PTP does not play a major role in LM BIK-induced cytochrome c release.

ER membranes have been shown to influence mitochondria by controlling the intracellular stores of divalent cations, mainly Ca2⁺ (30). Whereas a role of Ca2⁺ is unlikely in our in vitro system, since 1 mM EGTA was present in the incubation buffer and Ca2⁺-sensitive PTP does not appear to contribute to cytochrome c release (Fig. 6B), other cations such as Mg2⁺ could be important (31). However, 5 mM EDTA did not modulate the cytochrome c release activity of the HA-BIK S9 donor fraction (Fig. 6A). Thus, the BIK-induced cytochrome c release pathway observed here is unlikely to be mediated by an effect on the levels of free Ca2⁺ or Mg2⁺.

LM BIK-induced Cytochrome c Release Requires the LM in Addition to a Cytosolic Factor Independent of BAX—Since infection of H1299 cells with Ad HA-BIK promotes BAX insertion into mitochondrial membrane (Fig. 2B) and since regulated BAX insertion into mitochondrial membrane is a potential effector of BIK-induced cytochrome c release (7, 8), its role in the in vitro system was investigated. As shown in Fig. 7A, S9 extract from Ad-HA-BIK-infected cells but not from control cells caused BAX to become alkali-resistant. Since S9 Ad BIK contains very little BAX (Fig. 7B, Input H1299 S9 Ad BIK), the origin of alkali-resistant BAX is presumably that which is associated with the acceptor HM (see Fig. 2B, lane 2). To better assess the contribution of BAX, therefore, we prepared HM
from BAX-null liver. Both BAX−/− and BAX+/− acceptor HM could support cytochrome c release by H1299 S9 Ad BIK (data not shown). In addition, when using limiting concentration of the donor S9 that marginally cause cytochrome c release by itself, cytochrome c release was achieved by adding S100 from either BAX−/− or BAX−/- mouse liver (Fig. 7B), whereas the S100 on their own failed to induce cytochrome c release (data not shown). Under all conditions tested, there was no translocation of BAX to mitochondria (Fig. 7B). Altogether, these results indicate that BIK requires the presence of a constitutive cytosolic component to induce cytochrome c release from mitochondria, which is not BAX.

Since the depletion of LM from H1299 S9 Ad BIK abrogated its capacity to induce cytochrome c release (Fig. 5C), LM is also a required constituent. In contrast, LM on its own failed to induce cytochrome c release (Fig. 7C). In addition, no cytochrome c release was observed when the donor S9 fraction was preincubated for 30 min at 37 °C, after which the LM was spun down and the resulting supernatant was used as the donor in the cytochrome c release assay (Fig. 7C), indicating that a sustained presence of the LM is required. Thus, a complex signaling pathway is initiated by BIK, requiring both LM and cytosolic constituents.

**DISCUSSION**

Recent evidence suggests that BH-3-only BCL-2 homologues induce apoptosis by binding to mitochondria and causing cytochrome c release, dependent on the effector proteins BAX and BAK (7, 8). A number of these BH-3-only members, like BID and BAD, become activated in response to death signals through structural changes to preexisting inactive conformers (9). In contrast, p53 stimulates the production of several constitutively active BH-3-only proteins, including BIK, Puma, and Noxa, each of which can autonomously induce mitochondrial dysfunction and cell death. While Noxa and Puma have been reported to influence mitochondrial integrity directly (14–16), we show here that BIK can stimulate mitochondrial release of cytochrome c from a location at the ER. This is the first demonstration of a canonical BH-3-only member of the BCL-2 family initiating an apoptotic signaling pathway from this organelle.

A significant proportion of both overexpressed and endogenous BIK was found to co-localize with the ER marker calnexin, both by immunofluorescence in KB epithelial cells and by biochemical fractionation in H1299 cells. Although a second pathway involving mitochondrial BIK cannot be excluded, results from both in vitro reconstitution and the demonstration that FLAG-BIK-b5TM can induce cytochrome c release from mitochondria in vivo during cell death argue that ER-localized BIK is functional. Of note, however, we have found that APAF-1−/− cells are resistant to BIK-induced caspase activation and cell death but that BIK still induces cytochrome c release from their mitochondria (not shown). This result confirms that BIK operates upstream of cytochrome c release in an obligate APAF-1-mediated death pathway, as previously suggested by the use of a caspase-9 dominant negative (32).

The relationship between induction of ER (LM)-localized BIK and release of cytochrome c from mitochondria was studied in an in vitro system in which an S9 fraction from Ad HA-BIK H1299 cells was incubated with an HM fraction from control cells lacking BIK. It can be concluded from these experiments that ER-localized BIK triggers a cytochrome c-releasing activity that is dependent on the BIK BH3 domain as well as on the presence of both LM and cytosol. In this in vitro system, ER BIK did not dissociate from the LM to impose its activity by direct binding to mitochondria. In addition, cytochrome c release was not influenced by the PTP inhibitor BKA and was independent of BAX translocation/insertion into mitochondrial membrane. Although regulated targeting of BAX is one way in which mitochondrial dysfunction can be coupled to an upstream death signal, the lack of dependence may reflect the redundancy provided by the BAX homologue BAK, which is constitutively present in the mitochondrial outer membrane (8, 24). Collectively, however, our results suggest a complex pathway initiated by BIK, in which BIK regulates mitochondrial dysfunction through both ER and cytosolic factors independent of cytosolic BAX.

BIK was discovered through a search for proteins that interact with antiapoptotic BCL-2 proteins and was subsequently shown to readily co-immunoprecipitate with BCL-2 and BCL-XL (18, 19, 33). These antiapoptotic BCL-2 homologues have been shown to be associated with ER and nuclear membranes in addition to their mitochondrial location (10). The ratio of proapoptotic BIK and antiapoptotic BCL-2 homologues in the ER, therefore, may influence the ER pathway that regulates mitochondrial integrity. Although a pathway involving Ca2+ is an obvious candidate, neither this cation nor Mg2+ appears to be involved in the pathway described here. Of particular note, however, we also found that excess BCL-2 in the acceptor HM blocked cytochrome c release after stimulation with S9 Ad BIK, indicating that BCL-2 can also function downstream on the BIK pathway.

In conclusion, our data identify BIK as initiating a pathway...
from its location in the ER that stimulates cytochrome c release from mitochondria and that would be activated upon the induction of BIK protein by p53. This p53 pathway is distinct from other p53-induced targets such as the mitochondrial BH3-only proteins Noxa and Puma, suggesting yet another level at which p53-dependent apoptosis is controlled.

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