The BH3-only protein Bik/Blk/Nbk inhibits nuclear translocation of activated ERK1/2 to mediate IFNγ-induced cell death

Yohannes A. Mebratu,1 Burton F. Dickey,2 Chris Evans,2 and Yohannes Tesfaigzi1

1Lovelace Respiratory Research Institute, Albuquerque, NM 87108
2Department of Pulmonary Medicine, M.D. Anderson Cancer Center, Houston, TX 77030

Introduction

Although it is well established that IFNγ causes cell death in a variety of cell types (Deiss et al., 1995; Ossina et al., 1997; Wen et al., 1997; Ruiz-Ruiz et al., 2000; Trautmann et al., 2000; Horiuchi et al., 2006), the signal transduction downstream of STAT1 remains largely unknown (Barber, 2000). Unraveling the role of IFNγ in apoptosis remains a challenge because IFNγ may prime cells to apoptosis and through induction of many genes can concomitantly elicit an antiproliferative and a proliferative state (Xiang et al., 2008). The decision between life and death may depend on possible costimuli or the cell type. Enhanced expression and translocation of Diablo into the cytosol play a critical role in the promotion of IFNγ-induced apoptosis of IFNγ-sensitive B cells (Yoshikawa et al., 2001). Th1 cells that secrete high levels of IFNγ are more susceptible to activation-induced cell death than Th2 cells because Th2 cells express Fas-associated phosphatase, FAP-1 (Zhang et al., 1997). In keratinocytes, IFNγ induces apoptosis via increasing expression of Fas receptor (Trautmann et al., 2000), whereas the Fas ligand–Fas receptor pathway is not involved in the IFNγ-induced death of primary human airway epithelial cells (AECs [HAECs]; Shi et al., 2002; Trautmann et al., 2002). IFNγ induces cell death in AECs (Tesfaigzi, 2006) to remove hyperplastic epithelial cells after inflammation-induced epithelial cell hyperplasia by activating STAT1 (Shi et al., 2002), translocating Bax to the ER, and releasing ER calcium (Tesfaigzi et al., 2002; Stout et al., 2007). Disruption of the IFNγ-induced elimination of hyperplastic epithelial cells can be the source for chronic mucous secretions in asthma (Shi et al., 2002; Pierce et al., 2006) or for neoplastic growth over prolonged periods (Youn et al., 2005).

The Bcl-2 family of proteins consists of members with three to four Bcl-2 homology (BH) regions such as the pro-apoptotic proteins Bax and Bak (Lindsten et al., 2000) and the antiapoptotic members such as Bcl-2, Bcl-xL, and MCL-1. The interactions of these proteins are an essential gateway required for cell death in response to diverse stimuli (Wei et al., 2001) and under a wide variety of circumstances, suggesting that they act at a central control (CT) point in the pathway to apoptotic cell death (Adams and Cory, 1998; Cryns and Yuan, 1998;
Thornberry and Lazebnik, 1998). Another group of Bcl-2 family members contains only the BH3 motif and displays some selectivity for multiple domain Bcl-2 members (Oda et al., 2000; Letai et al., 2002) and provides a link between various cell death initiators and the execution machinery of apoptosis (Coulas et al., 2002; Opferman and Korsmeyer, 2003). BH3-only proteins inactivate the antiapoptotic proteins and allow activation of the multidomain proapoptotic members Bax and Bak (Cheng et al., 2001; Naik et al., 2007; Shimuzu et al., 2007; Willis et al., 2007). The proapoptotic activity of BH3-only molecules is kept in check by either p53-dependent transcriptional CT (Villunger et al., 2003), posttranslational modification (Verma et al., 2001; Lei and Davis, 2003), or by binding to the dynemic light chain in myosin V filamentous actin and thereby being sequestered from binding to Bcl-2 (Puthalakath et al., 2001; Day et al., 2004).

Our goal for this study was to further characterize the IFNγ-induced cell death in AECs by identifying the BH3-only proteins involved in this pathway. Bik/Bik/Nbk was consistently induced by IFNγ, and its expression induced cell death. Loss of Bik but not p53, Bim, or Bak conferred resistance to IFNγ but not to thapsigargin-induced cell death. Primary mouse AECs (MAECs) from p53- but not bik-deficient mice were protected from DNA damage–induced cell death. We demonstrate that the conserved Leu residue within the BH3 domain of Bik is crucial for its cell death–inducing activity by interacting with and suppressing the nuclear localization of phospho–extracellular regulated kinase 1/2 (ERK1/2) in MAECs and HAEcs. Furthermore, loss of Bik was accompanied by increased nuclear phospho-ERK1/2 and sustained epithelial cell hyperplasia in mouse airways, and blocking activated ERK1/2 with U0126 suppressed cell death in response to IFNγ treatment and Bik expression. Therefore, these experiments show that Bik is central in mediating IFNγ-induced cell death by retaining activated ERK1/2 in the cytosol in cultured AECs and during resolution of hyperplastic epithelial cells in mouse airways.

**Results**

**IFNγ induces Bik expression to elicit cell death in AECs**

We have previously shown that proliferating but not resting HAEcs undergo apoptosis in response to IFNγ (Shi et al., 2002) and that Bax plays a role in this cell death pathway (Tesfaigzi et al., 2002; Stout et al., 2007). To identify which of the BH3-only proteins initiate IFNγ-induced cell death upstream of Bax, primary HAEcs and AALEB cells, a cell line derived from HAEcs, were treated with IFNγ for 12, 24, 48, 72, and 96 h, and the changed expression of all the BH3-only members of the Bcl-2 family of proteins was screened using quantitative RT-PCR. Significant induction was consistently observed for Bik, but Puma, Hrk, Bid, and Bad remained unchanged. Bik mRNA levels were induced at 24–96 h (Fig. 1 A), and Bik protein levels were increased at 24 h after IFNγ treatment (Fig. 1 B) and remained elevated over 96 h (not depicted).

To determine whether Bik expression requires STAT1 activation, STAT1+/− and STAT1−/− MAECs were treated with IFNγ for 24 h, and cell extracts were analyzed for Bik expression. Results showed that Bik expression was significantly reduced in STAT1−/− compared with STAT1+/− MAECs (Fig. 1 C). Similarly, compared with Flag-expressing HBEC-2 cells, a significant reduction in Bik expression was observed in cells expressing a Flag-tagged dominant-negative construct for STAT1 that was previously shown to suppress IFNγ-induced cell death (unpublished data). However, p53, which has been shown to be important for inducing Bik (Han et al., 1996; Mathai et al., 2002), was not perceptibly affected in these cells (Fig. 1 C).

**The BH3 domain of Bik is crucial for inducing death in proliferating epithelial cells**

To investigate the role of Bik and the importance of the BH3 domain in inducing cell death, we expressed Bik adenoviral...
expression vector for Bik (Ad-Bik), mutant Bik (Ad-BikL61G), or adenoviral expression vector for GFP (Ad-GFP) in the immortalized AEC line, HBEC-2, and HAECs using an adenoviral expression system at an MOI of 100 (Fig. 1 D). Ad-Bik expression reduced the number of HBEC-2 cells significantly (Fig. 1 E) compared with Ad-BikL61G- or Ad-GFP-infected cells with an equal amount of MOI. As was previously observed for IFNγ (Shi et al., 2002), the ability of Bik to induce cell death was sensitive to cell growth conditions because confluent cultures of HBEC-2 cells or HAECs were unaffected by Ad-Bik infection, even though Bik expression was evident by Western blot analysis (unpublished data). To further investigate the role of Bik in IFNγ-induced cell death, IFNγ-induced Bik expression was suppressed in HAECs by infecting with a retroviral expression vector for Bik short hairpin RNA (shRNA), whereas CTs were infected with empty vector (Fig. 1 F). Suppression of IFNγ-induced Bik expression resulted in a significantly increased number of cells compared with cells infected with CT retrovirus (Fig. 1 F).

Overall, these studies showed that IFNγ induces cell death in AECs through Bik, although a previous study using bik−/− mice had shown that Bik has no role in hematopoietic cell death (Coulta et al., 2004). To determine whether Bik deficiency confers resistance to IFNγ-induced AEC death, we isolated MAECs from bik−/− mice and placed them in culture on Transwell membranes. As expected, Bik expression was induced by recombinant murine IFNγ at 24 h in bik−/− but not in bik+/− MAECs (Fig. 2 A). Consistent with the idea that IFNγ requires Bik to induce cell death, IFNγ significantly reduced the number of MAECs from wild-type but not from bik−/− mice (Fig. 2 B). MAECs from p53−/− mice died similarly to those from wild-type mice, whereas bim−/− or bax−/− MAECs appeared to be even more susceptible to IFNγ-induced cell death. IFNγ-induced Bik expression in p53−/− MAECs (Fig. 2 C), suggesting that p53 is not a crucial player in the IFNγ-induced cell death process.

We next determined whether Bik deficiency affected the response of MAECs to proapoptotic stimuli other than IFNγ. Loss of Bik did not confer any protection on MAECs undergoing apoptosis after exposure to the DNA-intercalating agent adriamycin, whereas p53−/− MAECs were completely protected (Fig. 2 D). Bik+/− and bik−/− MAECs were also equally susceptible to thapsigargin (Fig. 2 E). As was previously reported that HAECs are resistant to FasL (Hamann et al., 1998; Shi et al., 2002), treatment with FasL did not affect both bik−/− and bik+/− MAECs (unpublished data). Therefore, loss of Bik did not sensitize...
MAECs to FasL-induced cell death. However, consistent with the described experiments, Bik deficiency completely protected MAECs from IFNγ observed over a period of 48 h, whereas ~50% of bik+/− MAECs died over this time period (Fig. 2 F).

To further confirm that Bik is crucial for IFNγ-induced cell death, we reintroduced either wild-type Bik or mutant Bik into bik+/− MAECs using adenoviral infection and found significant cell death in bik+/− MAECs by expressing wild-type but not mutant Bik (Fig. 2, G and H).

Translocation of phosphatidylserine from the inner leaflet of the cell’s membrane to the outer leaflet is an early event in apoptotic cells that allows binding to annexin V (Vermes et al., 1995). Treating HBEC-2 cells with IFNγ for 48 h consistently showed a significant increase of cells that are positive for pro-cathepsins B and D (Fig. 3 C) and annexin V positivity (Fig. 3 D) significantly increased expression of cathepsins B and D was analyzed by RT-PCR. Both cathepsins were reproducibly induced by both IFNγ and Ad-Bik but not by Ad-GFP or Ad-BikL61G or in untreated CTs (Fig. 3 B). In addition, treatment with IFNγ increased expression of cathepsins B and D (Fig. 3 C) and annexin V positivity (Fig. 3 D) significantly in bik+/− compared with bik+/− MAECs, further confirming that Bik is a central mediator for this cell death pathway.

**Bik blocks nuclear translocation of activated ERK1/2 to cause cell death**

A previous study showed that IFNγ-induced cell death of oligodendroglial cells requires ERK1/2 activation (Horiuchi et al., 2006). To determine the mechanisms of how Bik mediates IFNγ-induced cell death, extracts from HBEC-2 cells treated with IFNγ for 0, 1, 4, 8, 24, and 48 h were subjected to Western blot analysis. ERK1/2 was significantly activated 24 h after IFNγ treatment (Fig. 4 A), but the extent of ERK1/2 activation was similar in bik+/− and bik+/− MAECs when treated with IFNγ for 24 h and Bik expression was evident (Fig. 4 B), suggesting that Bik was not mediating ERK1/2 activation. Previous studies had demonstrated that nuclear ERK activation causes cells to proliferate, although cytosolic ERK is associated with cell death (Lai et al., 2002; Chen et al., 2005). Therefore, we examined the distribution of phospho-ERK1/2 in IFNγ-treated bik+/− and bik+/− MAECs and found that activated ERK1/2 was reduced in the
nuclear extract of bik⁻/⁻ compared with bik⁻/⁺ MAECs (Fig. 4 C).

To further confirm that Bik overexpression inhibits nuclear translocation of ERK1/2, primary MAECs were infected with nothing as a CT, Ad-Bik, Ad-BikL61G, or Ad-GFP, and the cytosolic and nuclear extracts were analyzed by Western blotting 48 h later. We had previously observed that leaving the medium unchanged for 48 h caused sustained activation of ERK1/2 in HAECs (unpublished data). Although phospho-ERK1/2 was detected in the cytosolic extracts from CT and Ad-Bik⁻/⁻ infected cells, phospho-ERK1/2 was absent in the nuclear fraction of Ad-Bik⁻/⁻ infected HAECs but present in CT cells transfected with Ad-GFP and Ad-BikL61G⁻/⁻ infected cells (Fig. 4 D). To investigate whether Bik binds to activated ERK1/2 to inhibit its nuclear translocation, we performed immunoprecipitation assays with proteins extracted from HBEC-2 that were infected with either Ad-GFP, Ad-Bik, or Ad-BikL61G were immunoprecipitated with anti-Bik antibody. The cell lysates (input) and immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting using antibodies to Bik, phospho-ERK1/2, and total ERK1/2. (F) Representative photomicrographs and quantification showing that a higher percentage of MAECs from bik⁻/⁻ compared with bik⁻/⁺ mice displays nuclear localization of activated ERK1/2. MAECs were treated with 50 ng/ml murine recombinant IFNα/β for 24 h, fixed in paraformaldehyde, and immunostained for phospho-ERK1/2. The percentage of nuclei with phospho-ERK was quantified from three independent experiments. Bar, 10 μm. (G) HBEC-2 cells were infected with 100 MOI Ad-Bik and were cotreated with 1 μM U0126, the ERK-specific inhibitor. Cells infected with Ad-Bik showed a 60% decline in total cell number, and this decline was diminished when the HBEC-2 cells were cotreated with 0.1 or 1 μM U0126. The corresponding Western blot of proteins extracted from cells infected with Ad-Bik and treated with U0126 to suppress ERK1/2 activation. (H) Cell counts and Western blot analysis of protein extracted from HBEC-2 cells that were left untreated or treated with IFNα either alone or with U0126. Treatment with 1 μM U0126 did not affect cell growth. NT, not treated. Error bars indicate group means ± SEM (n = 4 different treatments per group). *, P < 0.05; significantly different from Ad-Bik⁻/⁻ infected cells.
U0126 (Fig. 4 G). Western blot analysis showed that in the presence of U0126, the levels of activation of cytosolic ERK1/2 were dramatically reduced in AECs infected with Ad-Bik (Fig. 4 G). Similarly, U0126 significantly attenuated IFNγ/H9253-induced reduction of cell numbers and suppressed IFNγ/H9253-induced cytosolic ERK1/2 activation (Fig. 4 H). Together, these experiments demonstrate that IFNγ/H9253 through Bik inhibits nuclear translocation of activated ERK1/2 and that cytosolic ERK1/2 is proapoptotic.

Bik mediates removal of hyperplastic epithelial cells in mouse airways

Repeated exposure to allergen results in proliferation and epithelial cell hyperplasia associated with the mucous phenotype; is detected in pull-down products from Ad-Bik– but not from Ad-BikL61G– or Ad-GFP–infected HBEC-2 cells (Fig. 4 E), suggesting that Bik directly interacts with phospho-ERK1/2 to sequester ERK1/2 in the cytoplasm.

Immunofluorescence staining showed that the percentage of cells with nuclear phospho-ERK1/2 was significantly higher in bik−/− MAECs compared with that observed in bik+/− MAECs after 24 h of IFNγ treatment (Fig. 4 F). These results demonstrate that expression of Bik suppresses nuclear translocation of activated ERK1/2 and that the conserved Leu residue in the BH3 domain of Bik is crucial for the inhibition of translocation. Activated ERK1/2 in the presence of Bik was proapoptotic because Ad-Bik–induced cell death was suppressed by the ERK1/2 inhibitor U0126 (Fig. 4 G). Western blot analysis showed that in the presence of U0126, the levels of activation of cytosolic ERK1/2 were dramatically reduced in AECs infected with Ad-Bik (Fig. 4 G). Similarly, U0126 significantly attenuated IFNγ-induced reduction of cell numbers and suppressed IFNγ-induced cytosolic ERK1/2 activation (Fig. 4 H). Together, these experiments demonstrate that IFNγ through Bik inhibits nuclear translocation of activated ERK1/2 and that cytosolic ERK1/2 is proapoptotic.

**Bik mediates removal of hyperplastic epithelial cells in mouse airways**

Repeated exposure to allergen results in proliferation and epithelial cell hyperplasia associated with the mucous phenotype;
however, when mice are continuously exposed for periods beyond 10 d, epithelial cell hyperplasia decreases (Tesfaigzi et al., 2002). Our previous experiments showed that IFNγ and STAT1 signaling are central for the resolution of allergen-induced epithelia cell hyperplasia (Stout et al., 2007). Because Bik was central for IFNγ-induced AEC death, we investigated whether this resolution process would be abrogated in IFNγ−/− and bik−/− mice. Interestingly, epithelial cell hyperplasia in IFNγ−/− airways after 5 d of allergen exposure was reduced compared with wild-type and bik−/− mice, suggesting that IFNγ plays a role in the allergen-induced proliferation of airway cells (Fig. 5, A and B). However, as expected, resolution of epithelial cell hyperplasia was abrogated in both IFNγ−/− (Fig. 5 A) and bik−/− (Fig. 5, B and C) mice during 15 d of allergen exposure, although it was significantly reduced in wild-type mice. Furthermore, the number of mucous cells per millimeter of basal lamina was significantly reduced in bik−/− mice compared with bik−/− mice during 12 and 15 d of allergen exposure (Fig. 5, D and E), confirming that Bik is central for IFNγ-induced killing in mouse airways as was observed in cultured MAECs. The role of Bik in inhibiting nuclear translocation of ERK1/2 was investigated by assessing the distribution of phospho-ERK1/2 in lung tissues of bik−/− mice exposed to allergen for 5 d but in significantly higher percentages in bik−/− mice at 5, 12, and 15 d of allergen exposure. Nuclear phospho-ERK1/2 was detected in airway cells of both bik−/− and bik−/− mice exposed to allergen for 5 d but in significantly higher percentages in bik−/− airways at 12 and 15 d of exposure (Fig 5 F). Interestingly, primarily the mucus-containing cells showed immunostaining for nuclear phospho-ERK1/2. Total ERK1/2 was found to be distributed similarly in both bik−/− and bik−/− mouse lungs after exposure to allergen for 5, 12, or 15 d. These findings show that Bik expression suppresses nuclear translocation of phospho-ERK1/2 in airways when resolution of hyperplastic epithelial cells occurs.

Discussion

IFNγ-induced Bik expression and cell death requires STAT1 but is independent of p53
Our studies suggest that IFNγ-induced activation of STAT1 causes Bik expression because IFNγ failed to induce Bik expression in STAT1−/− MAECs, and the same truncation mutant of STAT1 that reduced IFNγ-induced Bik expression also significantly reduced IFNγ-induced cell death of AECs (Stout et al., 2007). The findings from the present study in bik−/− mice together with our previous findings that allergen-induced airway epithelial hyperplasia is sustained in STAT1−/− mice (Stout et al., 2007) places STAT1 activation upstream of Bik. Whether STAT1 activation leads to increased Bik promoter activity or causes stabilization of Bik mRNA is currently unclear. Support for direct interaction and activation of the Bik promoter by STAT1 could be based on the presence of seven STAT-binding consensus sequences, TTTCNNNNAA (Bromberg and Chen, 2001), including two tandem sites within the core Bik promoter (Verma et al., 2000). However, our attempts to stimulate a Bik promoter luciferase construct with IFNγ were not successful, suggesting that other transcription factors or mRNA stability may play a critical role in the induction of Bik expression by IFNγ.

Overall, the findings suggest that although STAT1 may affect many signaling pathways in vivo, its activation requires Bik expression to channel the apoptotic effect.

Several observations show that the pathway by which IFNγ induces cell death does not involve the p53 pathway. (1) Resistance of bik−/− MAECs to IFNγ did not include the resistance to adriamycin that is mediated by DNA damage and p53 activation. (2) During IFNγ-induced cell death, p53 levels were not affected in AECs. (3) IFNγ induced Bik expression in p53−/− MAECs, suggesting that IFNγ does not require p53 to induce Bik expression and cell death. Similarly, others have reported that Bik mediates cell death in the p53-deficient H1299 cells and does not require p53 (Mathai et al., 2002).

Loss of Bik mediates resistance to IFNγ but not to other cell death inducers
IFNγ induces Bik expression that is localized to the ER (Mathai et al., 2005), translocates Bax to the ER, and elicits ER stress as shown by release of ER calcium stores (Stout et al., 2007) by JNK activation and induction of CHOP levels (unpublished data). Bax plays a role in IFNγ-induced cell death (Tesfaigzi et al., 2002), but bax−/− MAECs succumb to IFNγ treatment, suggesting that Bax can substitute for the loss of Bak as was reported previously (Lindsten et al., 2000; Wei et al., 2001). In fact, data from the present studies suggest that loss of Bik or Bax may sensitize MAECs more to IFNγ-induced cell death. Future studies need to investigate the role of Bim and Bax in the IFNγ-induced expression of Bik and/or the ER-stress pathway.

Bik−/− MAECs are resistant to IFNγ-induced ER stress, although they are sensitive to thapsigargin, a selective inhibitor of the ER-associated Ca2+-ATPase that allows Ca2+ to flow from the ER lumen into the cytoplasm. This sensitivity may be the result of IFNγ causing activation of ER stress and calcium release by mechanisms different from those induced by thapsigargin or because the ER-associated Ca2+-ATPase may be downstream of Bik. IFNγ and Bik-induced ER stress may be caused by the inactivation of GRP78 (BiP), an ER-associated protein that has antiapoptotic properties (Fu et al., 2007). Bik binds to GRP78 and allows the release of the critical transmembrane ER signaling proteins PERK, Ire1, and ATF6 (Xu et al., 2005). Protein shutdown caused by the bacterial toxin MazF or cycloheximide was also shown to require Bik to mediate cell death in TRex-293 cells (Shimazu et al., 2007). It is possible that inhibition of protein synthesis causes Bik expression by blocking the proteasome degradation system and results in massive ER stress through the GRP78 system.

Bik blocks nuclear translocation of activated ERK1/2 to cause cell death
In HAECS, IFNγ activated ERK1/2 24 h after treatment, which coincides with the time of Bik expression. ERK1/2 activation was also associated with IFNγ-induced death in oligodendroglial cells (Horiiuchi et al., 2006). Because ERK1/2 activation was similar in cytosolic extracts from bik−/− and bik−/− MAECs, we started to analyze whether Bik may affect the localization of phospho-ERK1/2. Both Western blot and immunofluorescence analyses confirmed that the presence of nuclear ERK1/2 was suppressed when Bik was expressed. Not only IFNγ- but also
stabilization-induced phospho-ERK1/2 was inhibited from translocating to the nucleus when Bik was present. The conserved Leu residue in the BH3 domain that was crucial for Bik-induced cell death was also crucial for interacting and inhibiting phospho-ERK1/2 translocation. Furthermore, inhibition of ERK1/2 using U0126 suppressed IFNγ- and Bik-induced cell death, suggesting that cytosolic ERK1/2 is proapoptotic, whereas nuclear ERK1/2 promotes growth in AECs. The biological consequence of ERK activation in a given cell may be determined by the cell-specific cytosolic or nuclear substrates. ERK is generally considered to be antiapoptotic (Kolch, 2005) but can function as a stimulator of apoptosis in cells expressing death-associated protein (DAP) kinase (Chen et al., 2005), a kinase that promotes the cytoplasmic retention of ERK1/2. DAP-kinase was isolated from HeLa cells as a mediator of IFNγ-induced cell death (Deiss et al., 1995; Levy-Strumpf et al., 1997), but its role in affecting the BH3-only proteins is not known. DAP-kinase is constitutively expressed in human HAECs and MAECs (unpublished data). Therefore, IFNγ-induced ERK1/2 activation and Bik expression may initiate a feedback loop that initiates DAP-kinase-mediated cytoplasmic retention of ERK1/2 to promote the amplification of proapoptotic signals. Bik was shown to interact with Bcl-2 (Boy et al., 1995), but this interaction is not sufficient for its apoptotic function (Elagovan and Chinnadurai, 1997). The present studies show that the proapoptotic function of Bik stems from its ability to inhibit nuclear translocation of phospho-ERK1/2.

The mechanisms of how Bik expression may induce expression of cathepsins B and D are unclear. Cathepsin B contains a TATA-less promoter but an E box that allows the formation of a transcription initiation complex involving the upstream stimulatory factors USF1 and ISF2 (Yan et al., 2003). Other transcription factors, including Ets1, Sp1, and EBS, regulate expression at two alternative promoters (Yan et al., 2000; Yan and Sloane, 2003). The TATA-containing promoter of cathepsin D is regulated by the hormone estrogen (Cavailles et al., 1993). Future studies will investigate whether Bik regulates expression of these mRNAs by reducing nuclear translocation of phospho-ERK1/2 that may have an inhibitory effect on these promoters or by increasing cytosolic phospho-ERK1/2 and prolonging mRNA half-life.

IFNγ can be proliferative but requires Bik to channel death

Exposure to allergen caused more AEC hyperplasia in bik−/− and wild-type mice compared with IFNγ−/− mice, suggesting that IFNγ plays a role in the proliferation of epithelial cells. However, once the hyperplastic stage was established, the resolution was abrogated in both IFNγ−/− and bik−/− mice. Such a double-sided effect for IFNγ has been previously reported (Hansen et al., 1999; Randolph et al., 1999; Xiang et al., 2008); however, this study demonstrates that IFNγ requires Bik to channel its cell death–inducing activity. So far, the murine Bik has only been suggested to represent a homologous equivalent of human Bik. The present studies show a role of human and murine Bik as the main mediator for IFNγ-induced cell death and, therefore, suggest that they are functionally homologous.

Although Bik is expressed in the liver, lung, heart, and kidneys and in granulocytes, macrophages, and developing as well as mature T and B lymphocytes (Coulthas et al., 2004), the bik−/− mice develop and age normally, suggesting that IFNγ-induced cell killing is not required during normal development under injury- and pathogen-free conditions. Loss of Bik conferred no protection to mature T and B cells from spontaneous death in culture, from treatment with dexamethasone or etoposide, or from cytokine starvation of mitogen-activated B cells (Coulthas et al., 2004). Bik inducing death in AECs rather than hematopoietic cells may result from airway cells expressing other genes such as DAP-kinase to facilitate Bik-mediated suppression of nuclear translocation of phospho-ERK1/2. The cell type–specific effect of BH3-only domain proteins is further supported by our previous studies showing that Bik, an essential initiator of apoptosis in negative selection of autoreactive thymocytes (Bouillet et al., 2002), and B cells (Enders et al., 2003), are not involved in the resolution of airway epithelial hyperplasia (Pierce et al., 2006).

The present studies show that loss of Bik or IFNγ leads to sustained epithelial cell hyperplasia after allergen-induced inflammation in mouse airways. In addition, proliferating rather than nonproliferating, resting AECs are prone to undergo cell death in response to IFNγ treatment or Bik overexpression. Therefore, Bik is ineffective in inducing cell death in confluent airway epithelial cultures (unpublished data). Primarily the mucus-containing cells showed nuclear phospho-ERK1/2 in lung tissues of mice exposed to allergen. Several studies have shown that after injury to the airway epithelium, a large proportion of the hyperplastic and cycling cells are secretary cells (Keenan et al., 1982a,b,c). Because mucous cells are the hyperplastic cells (Tesfaigzi et al., 2004), they may be by default susceptible to cell death and elimination in vivo during resolution. Susceptibility of proliferating cells to IFNγ or Bik-induced cell death may be a mechanism to selectively target hyperplastic epithelial cells that in airways usually represent mucus-producing cells (Lai et al., 2002). This selective elimination of only hyperplastic cells may ensure that resting AECs maintain the barrier function of the airway epithelium, may allow the CT of mucus production, and may prevent the development of cancerous lesions. Therefore, restoring Bik expression may be useful for eliminating hyperplastic airway cells. Bik was first identified as a protein that interacts with the adenoviral protein E1B 19K (Han et al., 1996; Mathai et al., 2002). The reason for E1B 19K or the viral homologue encoded by Epstein-Barr virus, BHRF1, singling out Bik for inhibition may be to sustain the viability of the host cells and promote replication and release of virus particles.

In summary, our analyses of HAECs and MAECs in culture and in airways of intact mice identify Bik as the central mediator of IFNγ-induced apoptosis. Furthermore, we provide evidence that Bik induces cell death by inhibiting nuclear translocation of activated ERK1/2 in a pathway that is independent of p53.

Materials and methods

Animals

Male-specific pathogen-free wildtype C57BL/6J and p53−/− mice were purchased from The Jackson Laboratory. Mice were housed in isolated cages under specific pathogen-free conditions. After a 14-d quarantine period, mice were acclimatized for 8 d and entered into the experimental...
protocol at 8–10 wk of age. The bax<sup>−/−</sup>, bim<sup>−/−</sup>, and bik<sup>−/−</sup> mice on C57BL/6 background were provided by S. Korsmeyer (Dana Farber Research Institute, Boston, MA) and A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia). Bax<sup>−/−</sup> and bax<sup>+/−</sup>, p53<sup>−/−</sup> and p53<sup>+/−</sup>, and bim<sup>−/−</sup> with bim<sup>+/−</sup> and bik<sup>−/−</sup> with bik<sup>+/−</sup> littermates were bred from the respective heterozygote mice at the Lovelace Respiratory Research Institute under specific pathogen-free conditions and genotyped as described previously (Coultais et al., 2004). STAT<sup>2−/−</sup> on C57BL/6 background were purchased from Taconic. All experiments were approved by the Institutional Animal Care and Use Committee and were performed at the Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International. Sensitization and exposure of mice to ovalbumin were performed as described previously (Tesfaijigzi et al., 2002). Preparation of lung tissues for histopathological examination was performed as described previously (Shi et al., 2002). Tissue sections were stained with Alcian blue (AB) and periodic acid Schiff or hematoxylin and eosin as described previously (Shi et al., 2002). The number of AB-positive cells per millimeter of basal lamina was quantified using a light microscope (BH-2; Olympus) equipped with the image analysis system (National Institutes of Health) as described previously (Harkema and Hotchkiss, 1991).

Cells
MAECs were harvested and cultured on Transwell membranes (Corning) after seeding with 4 × 10<sup>4</sup> or 9 × 10<sup>4</sup> cells as previously described (You et al., 2002). Cell viability was determined by trypan blue exclusion. Primary HAECs were purchased from Cambrex Bio Science Walkersville, Inc. The immortalized HAECs, AALEB, and HBEC-2 cells provided by S. Randell (University of North Carolina Chapel Hill, Chapel Hill, NC) and J. Shay (University of Texas Southwestern, Dallas, TX) were described previously (Lundberg et al., 2002; Ramirez et al., 2004). Cells were maintained in bronchial epithelial growth medium supplemented with growth factors as described previously (Shi et al., 2002).

Adenoviral expression vectors for Bik and Bik<sup>1610</sup> were provided by G. Shore (McGill University, Montreal, Quebec, Canada), and cells were infected as described previously (Mathai et al., 2002). The MAPK extracellular signal-regulated kinase inhibitor 4-diamino-2,3-dicyano-1,4-bis(2-amino-phenylthio)butadiene (U0126) was purchased from EMD. The doxorubicin hydrochloride (adriamycin) was purchased from Thermo Fisher Scientific, and phenylthio) butadiene (U0126) was purchased from EMD. The doxorubicin hydrochloride (adriamycin) was purchased from Thermo Fisher Scientific, and phenylthio) butadiene (U0126) was purchased from EMD. The doxorubicin hydrochloride (adriamycin) was purchased from Thermo Fisher Scientific, and phenylthio) butadiene (U0126) was purchased from EMD.

Retroviral silencing with Bik shRNA
Retroviral silencing vector encoding for Bik shRNA and the CT vector were purchased from Origene Technologies, Inc. The suppressive effect of the shRNA was established in HBEC-2 cells, and amplification and purification of plasmid DNA and packaging of the retroviral particles in Phoenix cells were performed as specified by the manufacturer’s instructions. After infection with Bik or CT shRNAs, HBEC-2 cells were treated with 50 ng/ml Bik-associating antibodies as described previously (Harris et al., 2005). Procedures for de- in Western blot analysis

Mice
Sponding band intensities for (Bio-Rad Laboratories). Band intensities were normalized with the correc- via densitometry using Fluor-S-Max Imager and Quantity One software and the ΔΔCT method as described previously (Schwalm et al., 2008). Because all results were de-

Assays (Applied Biosystems). For all reactions, CT values >37 were elimi-
forming amplification of nonamplified cDNA with TaqMan Gene Expression

map kinases extracellular signal-regulated kinase inhibitors (ERK1/2) and total ERK1/2 antibodies, respectively, after deprobing. Equal protein loading was confirmed by subsequent probing with the mouse monoclonal antibody against actin (Santa Cruz Biotechnology, Inc.).

Pull-down assay
A size X protein A immunoprecipitation kit (Thermo Fisher Scientific) was used to cross-link 50 μg of purified anti-Bik antibody to protein A beads using disuccinimidyl suberate as described by the manufacturer. Bik associated with Bik antibody on beads were eluted with 0.2 ml of ImmunoPure Elution buffer (Thermo Fisher Scientific) and analyzed by Western blotting and phosphoantibody and anti-ERK1/2 antibodies (Cell Signaling Technology). Total ERK1/2 was detected with anti-


Bik-associating antibodies as described previously (Harris et al., 2005). Procedures for de-

In Western blot analysis

For semiquantitative RT-PCR, total RNA was extracted from HBEC-2

References


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