ORIGINAL RESEARCH

Bik Mediates Caspase-Dependent Cleavage of Viral Proteins to Promote Influenza A Virus Infection

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Abstract

Influenza virus induces apoptosis in infected cells to promote viral replication by manipulating the host cell death signaling pathway. Although some Bcl-2 family proteins play a role in the replication of influenza A virus (IAV), the role of cell death pathways in the viral replication cycle is unclear. We investigated whether deficiency of the proapoptotic Bcl-2 family protein, Bik, plays a role in IAV replication. IAV replication was attenuated in mouse airway epithelial cells (MAECs) from $bik^{-/-}$ compared with $bik^{+/+}$ mice, as indicated by reduced viral titers. $Bik^{-/-}$ MAECs showed more stable transepithelial resistance after infection than did $bik^{+/+}$ MAECs, were less sensitive to infection-induced cell death, and released fewer copies of viral RNA. Similar results were obtained when Bik expression was suppressed in human airway epithelial cells (HAECs). $Bik^{+/+}$ mice lost weight drastically and died within 8 days of infection, whereas 75% of $bik^{-/-}$ mice survived infection for 14 days and were 10-fold less likely to die from infection compared with $bik^{+/+}$ mice. IAV infection activated caspase 3 in $bik^{+/+}$ but not in $bik^{-/-}$ MAECs. Cleavage of viral nucleoprotein and M2 proteins were inhibited in $bik^{-/-}$ MAECs and when caspase activation was inhibited in HAECs. Furthermore, Bik deficiency impaired cytoplasmic export of viral ribonucleoprotein. These studies suggest a link between Bik-mediated caspase activation and cleavage of viral proteins. Thus, inhibition of proapoptotic host factors such as Bik and downstream mediators of cell death may represent a novel approach to influenza treatment.

Keywords: Bcl-2 family protein; Bik; influenza A virus infection; caspase; mice

Clinical Relevance

This study identifies Bik as a novel host cell protein that plays an important role in influenza A virus replication. Because Bik promotes influenza A infection through caspase 3 activation and proper cytoplasmic export of viral RNPs, inhibiting Bik expression or caspase 3 activation may be a novel therapeutic approach to reducing influenza A infection.

Influenza A virus (IAV) infection is associated with 36,000 deaths and 226,000 hospitalizations annually, with losses in the tens of billions of dollars to the economy of the United States (1). Influenza infections pose serious challenges because of the lack of effective therapeutic interventions and the rapid evolution of viral genomes toward resistance. Therefore, understanding the molecular mechanism by which IAV replicates will help identify targets for effective antiviral drugs that are less susceptible to resistance.

Influenza virus induces apoptosis in infected epithelial, lymphocyte, and phagocytic cells (2) and mainly damages epithelial cells of the human respiratory tract (3). Although apoptosis is required for viral replication (3), how the cell death pathways interplay with viral replication is

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not well understood. IAV proteins can modulate host cell death by targeting cellular factors (4, 5). For example, although IAV nucleoprotein (NP) is implicated in viral pathogenesis and host range determination (6, 7), the viral proteins PB1-F2 localize to the mitochondrial membrane during infection to potentiate the apoptotic response in epithelial and fibroblastic cells through truncated BID signaling with proapoptotic Bcl-2 family protein members Bax and Bak (3, 8, 9).

The Bcl-2 family of proteins regulates apoptosis by promoting either cell survival or cell death. The prosurvival Bcl-2 family members include Bcl-2, Bcl-x_L, and Mcl-1 and contain up to four shared Bcl-2 homology (BH) domains. The proapoptotic proteins are divided into multi-BH domain members, such as Bax and Bak, containing BH1–3 domains, and the "BH3-only" members such as Bad, Bid, Bik, and Bim (8). Proapoptotic BH3-only proteins are upstream mediators of cell death, and their proapoptotic activity is tightly regulated by diverse transcriptional and post-transcriptional mechanisms (9). The BH3-only protein Bik, initially cloned from a yeast two-hybrid screen, interacts with viral and cellular antiapoptotic proteins such as adenovirus E1B-19K, EBV-BHRF1, BCL-2, BCL-x_L, and BCL-w (10). Bik localizes to the endoplasmic reticulum (ER) to induce mitochondrial apoptosis (11) and is crucial for regulating cell death in airway epithelial cells (AECs) (12, 13).

Bcl-2 decreases viral replication rates (14) by inhibiting IAV-induced cell death (15), because activation of caspase-3 during the cell death process is critical to the IAV life cycle (16). IAV NP acts as a shuttle for viral genomic segments from the nucleus to the budding sites at the plasma membrane, and localization during the virus replication cycle affects virus titers. The proapoptotic proteins promote IAV-induced apoptosis and virus replication (2, 16, 17) by facilitating proper shuttling of viral genomic segments from the nucleus to the budding sites at the plasma membrane. However, increased Bcl-2 expression (16, 18) that blocks caspase activation leads to improper nuclear retention of IAV and marked reduction in titers of infectious virus.

Host cell caspases play a role in the processing of influenza viral proteins and the maturation of viral particles (19). More recently, studies have shown that Bik mediates activation of caspases in cells infected with Epstein-Barr virus (20). Recent studies have shown that IAV-induced caspase activation causes a widening of nuclear pores to facilitate passive transport of viral ribonucleic protein (RNP) to ensure efficient production of infectious virus progeny (21). Inhibitors of host cell proteases have been used effectively to reduce viral replication and treat infection, such as in the case of HIV-1 infection (22). Several viruses, including IAV, express proteins that undergo host cell caspase cleavage (23).

Here we show, we believe for the first time, an intriguing link between the proapoptotic BH3-only protein Bik and susceptibility to IAV infection. Virus replication was attenuated and infection-induced cell death was reduced in $bik^{-/-}$ compared with $bik^{+/+}$ AECs. *In vivo*, $bik^{-/-}$ mice lost less body weight, survived longer, and were 10-fold less likely to die from infection compared with $bik^{+/+}$ mice. IAV increased Bik protein level and activated caspase 3. Bik mediated caspase 3 activation to promote cleavage of viral NP and M2 protein. Furthermore, silencing Bik in human AECs (HAECs) impaired cytoplasmic export of viral RNPs.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The $bik^{+/-}$ and $noxa^{+/-}$ mice on C57BL/6 background provided by Andreas Strasser (Walter and Eliza Hall Institute, Melbourne, Australia) were bred at the Lovelace Respiratory Research Institute and genotyped as described (24). All experiments were approved by the Institutional Animal Care and Use Committee and the Environmental Safety and Health Department of Lovelace Respiratory Research Institute.

Cells

Mouse AECs (MAECs) isolated from mouse trachea were cultured on plastic plate or Transwell membranes (Corning, New York, NY) as described previously (25). Immortalized HAECs, AALEB, provided by S. Randell (University of North Carolina, Chapel Hill, NC) were described previously (26).

Retroviral Silencing

Retroviral silencing vector encoding for Bik small hairpin RNA (shRNA) and the control vector were purchased from Origene Technologies (Rockville, MD) and were generated following the manufacturer's instruction as described (12). Details are provided in the online supplement.

Viral Growth and Titration

Influenza A/PR/8/34 strain was propagated in embryonated chicken eggs and titrated on Madin-Darby canine kidney cells as described (2). Infectious virus yields were analyzed from cell supernatants and cell lysates of IAV-infected cells using plaque assays as described (2). Viral titers in lung tissues were determined by 50% tissue culture infectious dose ($TCID_{50}$) as described (27). Details are provided in the online supplement.

Lung Histopathology

To analyze inflammation-associated lung damages, hematoxylin and eosin–stained lung tissues were evaluated in a blinded manner with a semiquantitative system as described previously (28). Details are provided in the online supplement.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

To amplify IAV M1 gene using quantitative reverse transcriptase–polymerase chain reaction, viral RNA was isolated using a QIAamp kit (QIAGEN, Hilden, Germany) and analyzed as described previously (29). The primers used are described in the online supplement..

Cell Death Assays

Cell viability in IAV-infected cells was assessed using a trypan blue exclusion assay as described (12). Apoptotic cells were stained with Annexin V-FITC conjugate (BD Biosciences, San Jose, CA) and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and were analyzed using BD FACSCanto Flow Cytometer (BD Biosciences) as described (30) using FlowJo analysis software (Tree Star, Ashland, OR).

Immunoblotting

Protein lysates were prepared and analyzed by Western blot as described previously (12). Antibodies used are described in the online supplement.

Immunostaining

Cell monolayers fixed in acetone-methanol were mounted in paraffin. To label IAV NP, H_2O_2-treated monolayer sections were incubated overnight at 4°C with rabbit anti-NP antibody (MAB5281, EMD Millipore, Darmstadt, Germany) followed by peroxidase-labeled goat antirabbit IgG (Pierce, Rockford, IL) for 1 hour. Cilia were labeled with antiacetylated α-tubulin antibody. The localization of viral NP was analyzed in HAECs grown on Lab-Tek-II slides as described (12). Details are provided in the online supplement.

Data Analysis

Data are expressed as the mean group value ± SEM and analyzed using SAS (Statistical Analysis Software Institute, Cary, NC). For the *in vivo* studies, differences in body weight were expressed as mean group value ± SEM. The survival rates of mice were analyzed using the log-rank (Mantel-Cox) test. When significant main effects were detected ($P < 0.05$), the Fisher least significant difference test was used to determine differences among groups.

Results

Bik Deficiency Suppresses IAV Replication in MAECs

The Bcl-2 family of protein is involved in IAV replication (2, 16, 17). We reported previously that Bik is a major inducer of apoptosis in the AECs (12). To investigate whether Bik contributes to IAV infection and replication, MAECs isolated from the trachea of WT, *bik*$^{-/-}$, and *noxa*$^{-/-}$ mice were driven to differentiation in air–liquid interface cultures and infected with an influenza A/PR/8/34 (H1N1) strain that has been adapted to mice for lethality. Noxa, another proapoptotic BH3-only protein of the Bcl-2 family, contributes to apoptosis induced by p53 overexpression or DNA damage in diverse cells. However, its apoptotic potential is variable; significant cell death occurs in some systems (31), but it is poorly apoptotic in other types of cells (32). Thus, MAECs from *noxa*$^{-/-}$ mice were used as controls to determine whether the reduced virus replication seen in *bik*$^{-/-}$ cells is specific to Bik deficiency or is a function of the BH3 domain. Immunostaining for viral NP showed that, although there was no significant difference in IAV NP positivity among WT, *bik*$^{-/-}$, and *noxa*$^{-/-}$ MAECs at 4 hours post infection (hpi) (*see* Figure E1A in the online supplement), the percentage of NP-positive cells decreased by ∼ 35% in *bik*$^{-/-}$ compared with WT and *noxa*$^{-/-}$ cells 24 hpi (Figure 1A). Plaque assay results from three independent studies showed consistently that infectious virus titers were reduced in *bik*$^{-/-}$ cells both in the apical wash (Figure 1B) and in cell extracts (Figures E1B and E1C) compared with the WT and *noxa*$^{-/-}$ cells. In addition, after infection, the *bik*$^{-/-}$ MAECs showed the lowest decline in transepithelial electric resistance, a measure of integrity of epithelial cell integrity that encompasses tight junctions and epithelial cell viability, as compared with WT or *noxa*$^{-/-}$ cells (Figure 1C). Microtubules are required for efficient epithelial tight junctions and influenza cellular egress (29). Consistent with findings for transepithelial electric resistance values, the microtubule integrity was disrupted more in WT than in *bik*$^{-/-}$ cells (Figure 1D).

The observed differences in virus titers may be caused by differences in viral genomic replication, assembly, or maturation. Analysis of the viral RNA released after infection of differentiated culture cells using primers and probes for the M1 gene of IAV by quantitative reverse transcriptase–polymerase chain reaction showed that fewer copies of viral RNA were detected in the supernatants of *bik*$^{-/-}$ cells compared with WT or *noxa*$^{-/-}$ MAECs (Figure 1E). Furthermore, reduced levels of viral proteins were detected in the cell lysates of *bik*$^{-/-}$ compared with *bik*$^{+/+}$ cells (Figure 1F). Thus, the differences in the virus titer between *bik*$^{+/+}$ and *bik*$^{-/-}$ cells may be caused by differences in genomic replication of IAV. These findings suggest that Bik deficiency confers resistance against efficient replication of the virus in AECs. Specificity of these responses to Bik was supported by deficiency of another BH3-only protein, Noxa, showing susceptibility similar to that of WT cells.

Lethality of Influenza A/PR/8/34 Infection in Bik$^{+/+}$ and Bik$^{-/-}$ Mice

Before investigating whether *bik*$^{-/-}$ compared with *bik*$^{+/+}$ mice are more resistant to infection, we established a viral burden that causes near lethality in WT mice. C57BL/6 mice were instilled with 0, 10, 100, or 1,000 plaque forming unit (pfu) IAV intranasally and were monitored daily over 14 days. IAV caused significant loss in body weight (Figure 2A) at doses of 100 and 1,000 pfu.

We chose the near-lethal dose of 500 pfu A/PR/8/34 virus (personal observation) to compare viral effects in *bik*$^{+/+}$ with those of *bik*$^{-/-}$ mice *in vivo*. Over a period of 14 days, *bik*$^{+/+}$ mice lost significantly more weight compared with *bik*$^{-/-}$mice (Figure 2B). Although seven of the eight *bik*$^{+/+}$ mice (>85%) died within 8 days, 75% of the *bik*$^{-/-}$ mice survived infection for 14 days. Analysis of the survival data using the log-rank (Mantel-Cox) test showed that the *bik*$^{-/-}$ mice survived for significantly more days after infection compared with the WT mice ($P < 0.005$) (Figure 2C) The hazard ratio for the *bik*$^{-/-}$ mice compared with the *bik*$^{+/+}$ mice was 0.102 (95% confidence interval, 0.02–0.5), suggesting that mice infected with IAV were ∼10-fold less likely to die when they were deficient in Bik. To determine whether decreased viral load correlates with reduced mortality in *bik*$^{-/-}$ mice, we used $TCID_{50}$ to measure the virus load in the lung tissues of mice 5 days after infection. Compared with that in *bik*$^{+/+}$ mice, the lung viral titer in *bik*$^{-/-}$ mice was ∼3 log lower (Figure 2D). Further evaluation of the HandE-stained lung tissues showed bronchointerstitial pneumonia characterized by fibrin exudation and cellular infiltrates investing the vascular connective tissues and peribronchial alveolar septa. Semiquantitative histopathological evaluation of lung inflammation and tissue damage was rated on the basis of the degeneration and necrosis levels of bronchi and bronchiolar epithelium, the infiltration of inflammatory cells, and alveolar degeneration. Consistent with the reduced morbidity and mortality and decreased virus load in the lung tissues, compared with *bik*$^{+/+}$ mice, *bik*$^{-/-}$ mice exhibited significantly less severe lung damage (Figure 2E).These *in vivo* results confirmed the observation that in cultured cells, Bik plays a significant role in the pathogenesis of IAV.

Bik Mediates IAV-Induced Cell Death and Cleavage of Viral Proteins in AECs

Infection of AALEB cells, a cell line derived from normal human bronchial epithelial cells, with the same IAV strain, A/PR/8/34, caused significant cell death compared with noninfected controls (Figures 3A and 3B). Although the

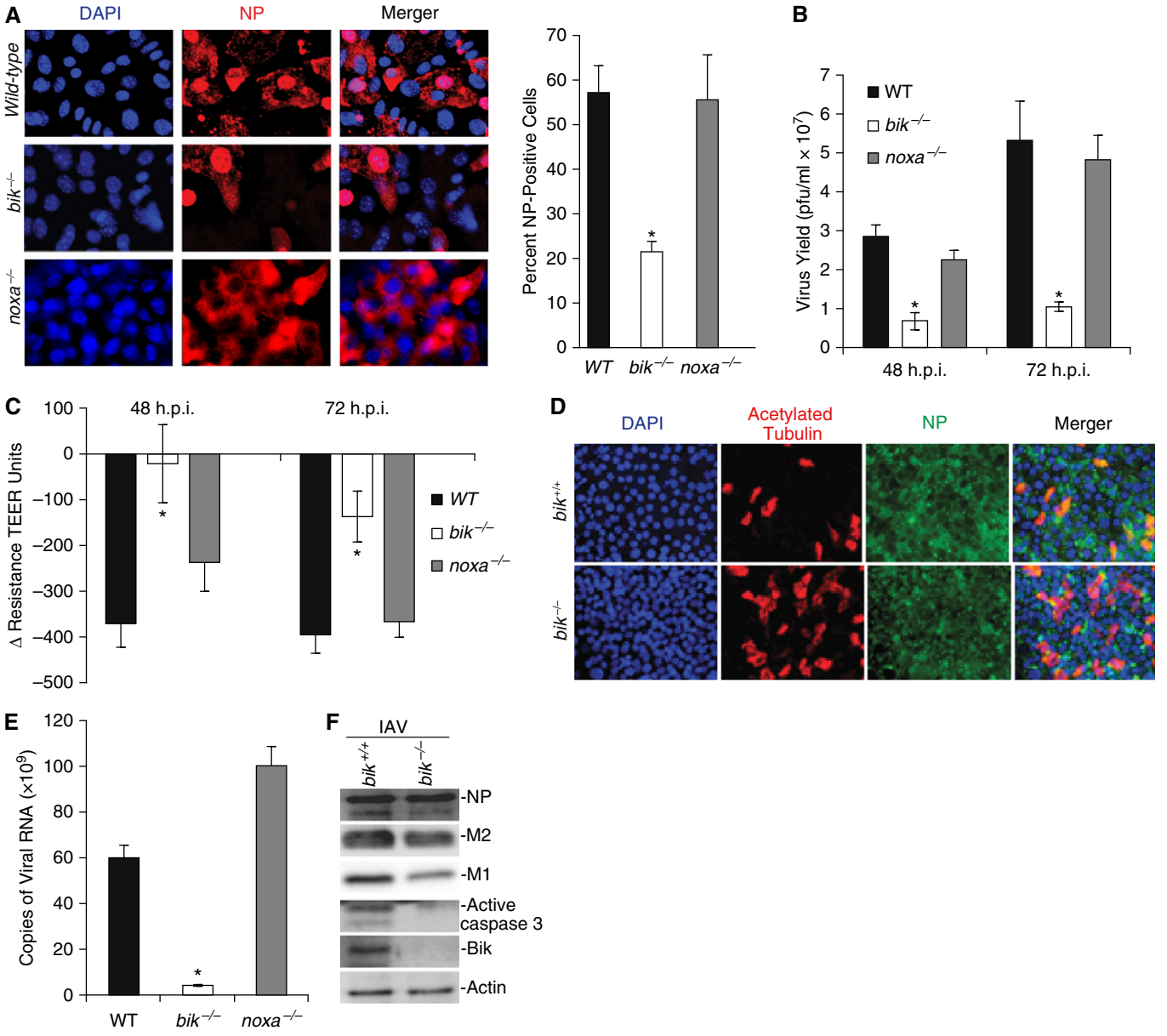

Figure 1. Bik facilitates influenza A virus (IAV) infection and increase of viral RNA and protein. Differentiated mouse airway epithelial cells (MAECs) from wild-type (WT) *bik*$^{-/-}$ and *noxa*$^{-/-}$ mice were infected with influenza A/PR/8/34 at 0.1 multiplicity of infection (MOI). (*A*) Cells were immunostained for viral nucleoprotein (NP) and analyzed with fluorescent microscopy 24 hours post infection (hpi). The percentages of NP-positive cells were quantified from three independent experiments. (*B*) Viral titer analyzed by plaque assay in the apical washes at 48 and 72 hpi. (*C*) Change in transepithelial electric resistance at 48 and 72 hpi. (*D*) Differentiated MAECs from *bik*$^{+/+}$ and *bik*$^{-/-}$ mice infected with 0.1 MOI IAV for 48 hours, fixed and stained for acetylated α tubulin (*red*), NP (*green*), and DAPI (*blue*). Cells were analyzed by fluorescent microscopy. (*E*) Differentiated MAECs from *bik*$^{+/+}$ and *bik*$^{-/-}$ mice were infected with 0.1 MOI IAV for 24 hours. Copies of the viral RNA released into the cell culture supernatant were quantified by quantitative reverse transcriptase–polymerase chain reaction using primers and probes for M1 gene. (*F*) *bik*$^{+/+}$ and *bik*$^{-/-}$ MAECs infected with 0.1 MOI IAV and protein lysates analyzed 24 hpi for the expression of viral proteins (NP, M2, M1), activated caspase 3, and Bik levels by Western blot. Data are expressed as mean ± SEM for three independent experiments. *$P < 0.05$. DAPI, 4',6-diamidino-2-phenylindole; TEER, transepithelial electric resistance.

proapoptotic proteins promote IAV replication (2, 16, 17, 33), the prosurvival Bcl-2 members decrease viral replication rates (14, 16). Therefore, we screened for changes in the expression of Bik, bad, Mcl-1, and Bcl-x_L. Bik was increased in expression 8 hpi, which coincided with signal transducers and activators of transcriptions (STAT)1 phosphorylation. The expression of Bik was significantly enhanced 16–48 hpi (Figures 3C and E2A). Bad was increased in expression at 16 hpi, and this expression was sustained at 24 and 48 hpi. Although IAV infection did not increase the expression of Mcl-1, compared with the control, Bcl-x_L was increased slightly at 8 hpi with no further increase at later time points (Figure 3C).

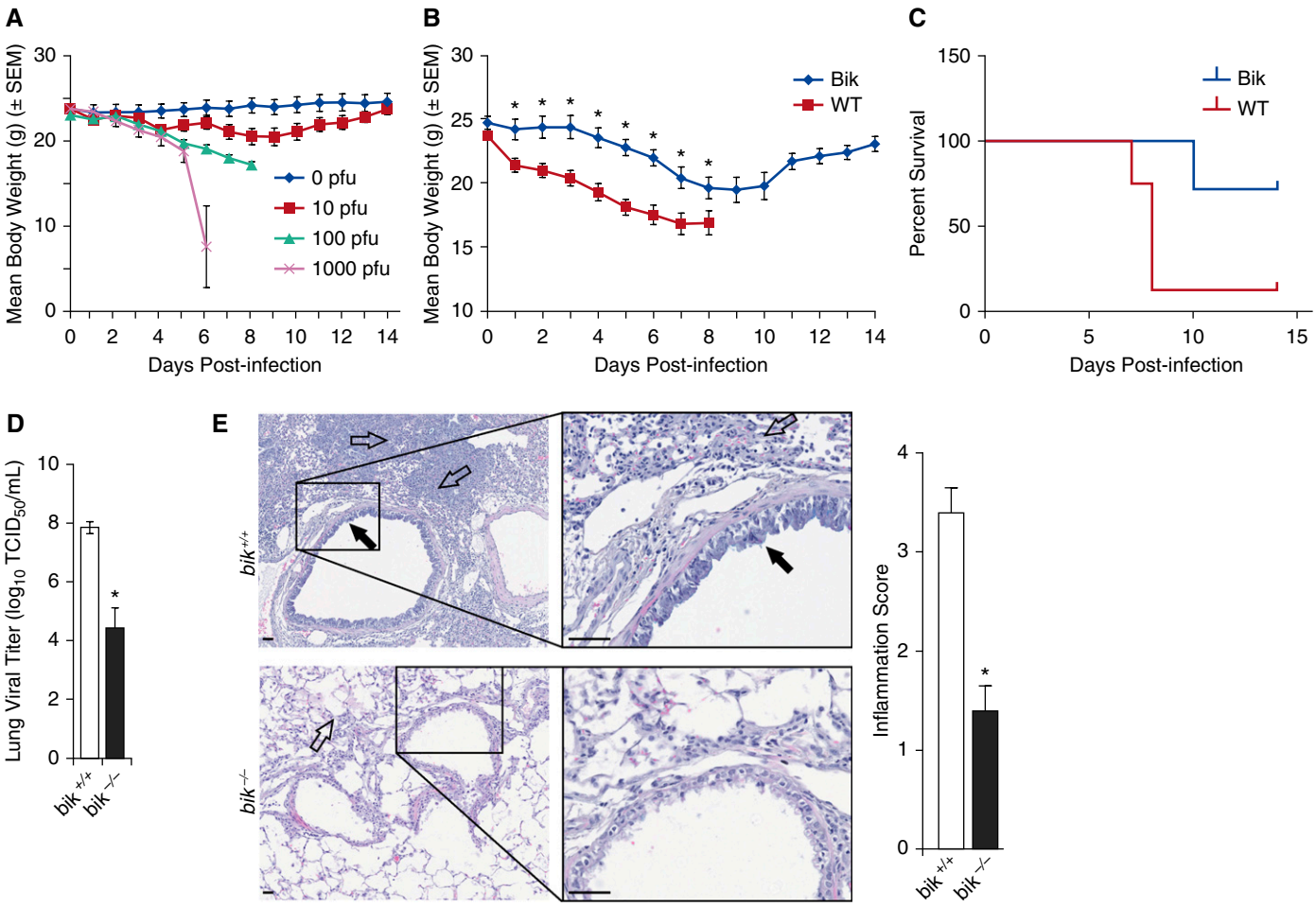

Figure 2. Bik-deficient mice are protected from IAV-induced morbidity and mortality. Mean changes in body weight (*A*) after infection of WT C57BL/6 mice with different dosage of influenza A/PR/8/34. Mice were instilled with 0, 10, 100, or 1,000 plaque-forming units (pfus) of IAV in 50 μl of PBS intranasally and monitored daily for a period of 14 days for changes in body weight and mortality. Mice were considered moribund when they lost 20% of their body weight and were killed. (*B* and *C*) Comparison of mean changes in body weight (*B*) and percent survival rates (*C*) between $bik^{+/+}$ and $bik^{-/-}$ mice infected with 500 pfus of IAV intranasally and monitored daily for a period of 14 days post infection ($n = 8$ per group). (*D*) Lung tissue viral titers of $bik^{+/+}$ and $bik^{-/-}$ mice on Day 5 after infection ($n = 5$). The viral titer was significantly reduced in the lung tissues of $bik^{-/-}$ mice compared with $bik^{+/+}$ mice. (*E*) Histopathology of HandE-stained lung tissues from $bik^{+/+}$ and $bik^{-/-}$ mice on Day 5 after infection. $bik^{+/+}$ mice displayed more severe inflammation than did $bik^{-/-}$ mice. The *open arrows* indicate inflammatory cell infiltration in the alveoli and septa; the *solid arrows* indicate hyperplasia and mucous cell metaplasia. *Scale bar* = 25 μm. Inflammation scores were evaluated as described in Materials and Methods ($n = 5$). *$P < 0.01$. $TCID_{50}$, 50% tissue culture infectious dose.

Bik is a major regulator of cell death in the AECs (12, 13). Infection of $bik^{+/+}$ and $bik^{-/-}$ MAECs with 0.1 multiplicity of infection (MOI) IAV showed that $bik^{-/-}$ MAECs were significantly more resistant to IAV-induced cell death as quantified by trypan blue exclusion assay (Figure 3D). Furthermore, when Bik was suppressed by targeted degradation of messenger RNA using shRNA (Figure 4A), AALEB cells were protected from IAV-induced cell death compared with control small hairpin RNA (shCTR)-expressing cells (Figure 3E), further confirming that Bik mediates IAV-induced cell death.

Translocation of phosphatidylserine from the inner to the outer leaflet of the cell's membrane is an early event in the life-cycle of apoptotic cells that allows binding to annexin V (34). Fluorescent-activated cell-sorting analyses of cells infected with 0.1 MOI IAV cells showed that IAV infection caused a significant increase in PI- and annexin V-positive cells in shCTR compared with small hairpin RNA for Bik (shBik)-expressing AALEB cells (Figure 3F, *upper panel*). The percentage of cells in early (Figure 3G), late (Figure 3H), and total annexin V positivity (Figure 3I), indicative of total cell death, was significantly reduced in shBik compared with shCTR-expressing cells. Together, these results confirm that Bik is a major mediator for the IAV-induced apoptotic pathway.

Caspase activation plays a major role in IAV pathogenesis (16, 18, 29, 35), and increased expression of BIK causes the release of cytochrome c from mitochondria to activate caspases (36). We also found that IAV infection caused cleavage of caspase 3 in MAECs (Figure 1F) and HAECS cells (Figures 4A and 4B), but caspase activation was inhibited in $bik^{-/-}$ MAECs (Figure 1F) and Bik knockdown HAECS cells (Figures 4A and 4B), both of which were resistant to virus-induced cell

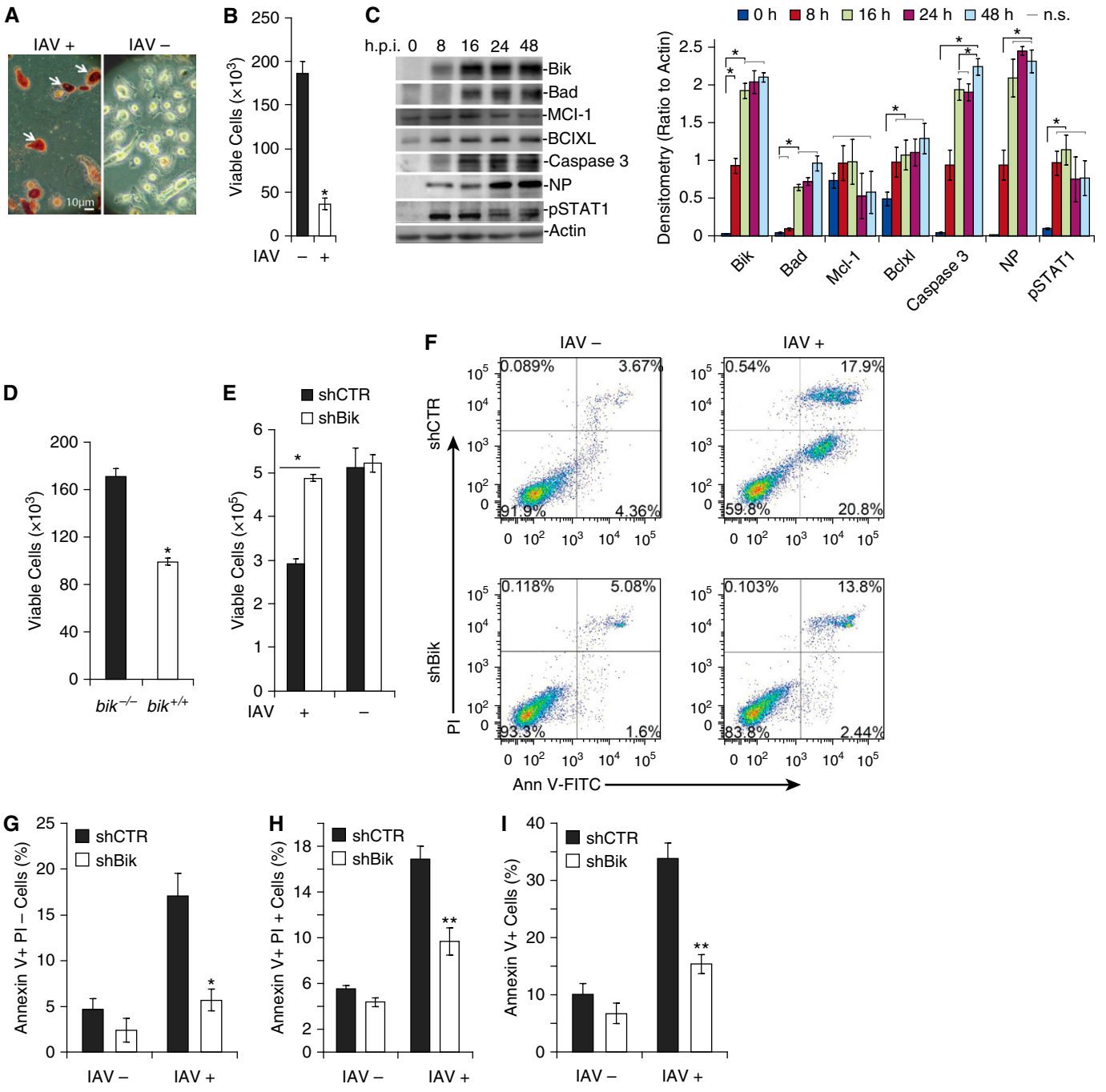

Figure 3. Influenza virus causes cell death in human airway epithelial cells (HAECs) in a Bik-dependent manner. HAECs plated on 6-well culture plate were infected with mock or 0.1 MOI influenza A PR/8/34 virus, and 48 hours later were stained with anti-IAV NP antibody (*A*) or viable cells quantified after staining with trypan blue (*B*). (*C*) HAECs were infected with 0.1 MOI influenza A PR/8/34 virus, and protein lysates were analyzed for the level of Bik, Bad, Mcl-1, Bclxl, pSTAT1, and viral NP proteins by Western blot at 0, 8, 16, 24, and 48 hours after infection. Densitometric quantification normalized to actin content was used to analyze the fold change. *$P < 0.01$. (*D*) Mouse airway epithelial cells from *bik*$^{+/+}$ and *bik*$^{-/-}$ mice plated on 6-well plate were infected with mock or 0.1 MOI IAV and quantified for cell viability 48 hours later, after staining with trypan blue. (*E*) HAECs stably expressing control small hairpin RNA (shCTR) or small hairpin RNA for Bik (shBik) were infected with 0.1 MOI influenza A/PR/8/34, and cells were quantified 48 hours after infection by trypan blue exclusion assay. (*F–I*) HAECs stably expressing shCTR or shBik were infected with 0.1 MOI IAV and stained with propidium iodide (PI) and Annexin V 48 hours later. Survival profile of the cells was analyzed by fluorescence-activated cell sorter. (*F*) Viable cells (Annexin V$^-$ and PI$^-$), early-stage apoptosis (Annexin V$^+$ and PI$^-$) and late-stage apoptosis (Annexin V$^+$ and PI$^+$) are represented in the *bottom left*, *bottom right*, and *top right quadrants*, respectively. Data for 10, 000 cells were collected in each case, and the percentages of the total population in these quadrants are shown. Representative figures of three independent experiments are shown. *Bar graphs* show the mean percentage of cells in early (*G*), late (*H*), and total (*I*) apoptosis from three independent experiments. *Scale bar*: 10 μm. *White arrows* in *A* indicate NP-positive cells. *$P < 0.001$; **$P < 0.0001$. Ann V-FITC, annexin five fluorescein isothiocyanate; n.s., no significant difference; pSTAT1, phosphorylated signal transducers and activators of transcription 1.

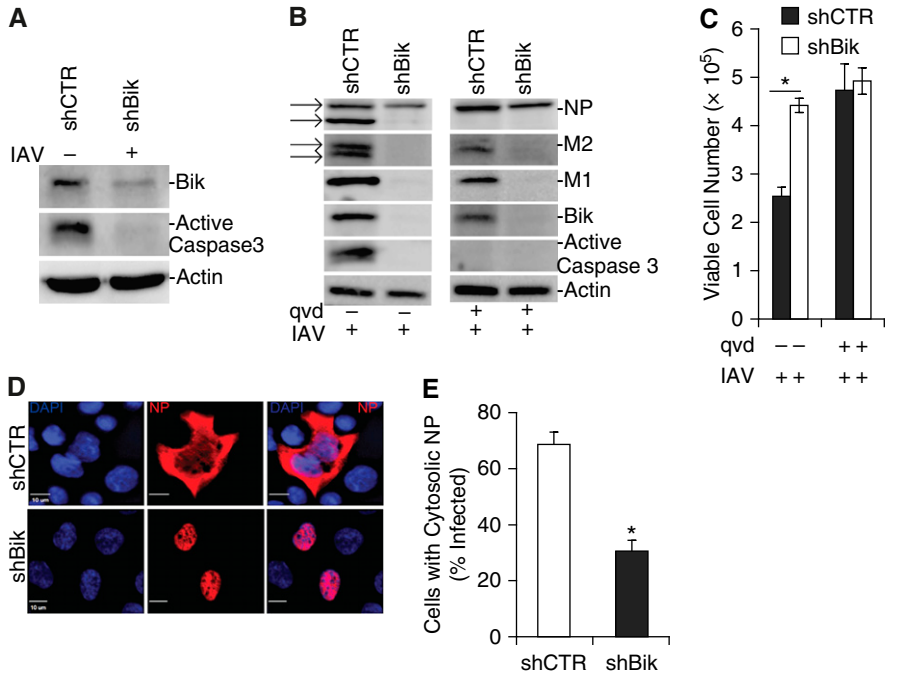

Figure 4. Bik mediates IAV-induced caspase 3 activation and cytoplasmic localization of viral RNP. (*A*) HAECs stably expressing shCTR or shBik were infected with 0.1 MOI of influenza A/PR/8/34 virus, and protein lysates were analyzed 24 hours after infection for Bik levels and caspase activation using antibodies to Bik and anti-active caspase 3 by Western blotting. (*B*) HAECs expressing shCTR or shBik were infected with 0.1 MOI influenza A/PR/8/34 in the presence and absence of 20 μM Q-VD, and protein lysates were analyzed for Bik, active caspase 3, and cleaved viral NP, M2, and M1 proteins by Western blotting. (*C*) Viable cells were quantified after staining with trypan blue. (*D*) HAECs stably expressing shCTR or shBik were infected with influenza A/PR/8/34 at 0.1 MOI and were subjected to immunostaining using a specific anti-viral NP antibody and secondary antibodies conjugated to Dylight-649 24 hours after infection. Cells were mounted with DAPI containing Fluormount-G for nuclear staining and analyzed with fluorescent microscopy. *Scale bar* = 10 μm. (*E*) Percentage of IAV-infected cells with NP localized to the cytoplasm. Experiments were done in triplicate, and the localization of viral NP in infected cells was quantified by counting at least 100 cells per experiment. *$P < 0.005$. qvd, quinoline-Val-Asp-difluorophenoxymethylketone; RNP, ribonucleoprotein.

death (Figures 3D–3F). These findings suggest that Bik mediates IAV-induced cell death through caspase activation.

Activation of the proapoptotic cascade has been suggested as playing a role in the processing of influenza viral proteins and the maturation of viral particles (19). Furthermore, cleavage of viral proteins by host cell proteases and protease cleavage motifs have been identified in proteins encoded by IAVs (18, 37, 38). Because IAV-induced activation of caspase 3 was inhibited in *bik*$^{-/-}$ MACEs and shBik HAECs, we tested whether viral proteins are cleaved in a Bik-dependent manner. Cell lysates prepared from shBik- and shCTR-expressing cells showed cleavage of NP and M2 proteins in shCTR, but not in shBik-expressing cells (Figure 4B). No perceivable cleavage of the M1 protein was observed in either shCRT- or shBik-expressing cells. The detected amount of viral proteins was reduced significantly in shBik compared with shCRT HAECs cells, with M1 and M2 proteins not visibly detected. In addition, not only cleavage of viral NP and M2 proteins, but also IAV-induced cell death, was suppressed when caspase activation was blocked by the pan-caspase inhibitor, Q-VD (Figure 4C). Together, the attenuation of viral propagation in *bik*-deficient cells may be explained by the reduced accumulation of viral proteins.

Cytoplasmic Export of the Viral RNPs Is Inhibited in Bik-Deficient Cells

Because IAV NP acts as a shuttle for viral genomic segments from the nucleus to the budding sites at the plasma membrane, we determined whether the lack of Bik signaling affects proper IAV NP localization. Interestingly, although efficient RNP migration to the cytoplasm was detected readily in shCTR-expressing cells at 24 hpi, cytoplasmic accumulation of the RNPs was inhibited in shBik-expressing cells, with 68% of infected cells showing cytoplasmic localization of viral RNP in shCTR-expressing cells compared with 30% in shBik-expressing cells (Figures 4D and 4E), suggesting the potential for impaired shuttling of viral RNP to the cytoplasm by Bik deficiency and reduced caspase activation.

Discussion

We found that loss of Bik is associated with impaired IAV replication in differentiated airway cultures and with increased resistance to IAV-induced morbidity and mortality in mice. Bik mediated intracellular cleavage of viral proteins and proper cytoplasmic transport of viral RNPs. Thus, we believe this is the first report documenting a direct link between the proapoptotic BH3-only protein Bik and IAV replication.

Infection of *bik*$^{-/-}$ compared with *bik*$^{+/+}$ MAECs resulted in a significant reduction in virus yield, accompanied by a reduction in the copies of viral RNA released from infected cells, suggesting that Bik affects viral genomic replication. Furthermore, the *bik*$^{-/-}$ mouse had a lower viral load in the lung tissues and milder inflammation compared with the WT mice. Thus, the reduction in morbidity and increased survival of *bik*$^{-/-}$ mice compared with *bik*$^{+/+}$ mice is a result of both reduced virus replication and milder inflammation in the lung tissues.

In the current study, although IAV caused an increase in the expression of Bik 8 hpi, which was significantly enhanced 16–48 hpi, the level of Bad started to increase at 16 hpi. Although Bad plays an important role in IAV infection (39), deficiency of Bik was sufficient to cause reduced viral titer and viral gene expression, reduced virus load and lung inflammation *in vivo*, and increased survival of mice. It is possible that Bik may be upstream of Bad to directly or indirectly affect Bad expression and Bad may play an important role downstream of Bik in IAV infection (39). These possibilities suggest a significant role played by Bik in IAV pathogenesis. We found previously that IFN-γ increased the expression of Bik in a STAT1-dependent manner to cause cell death in the AECs

(12). In the current study, IAV-induced STAT1 activation preceded increased Bik expression, suggesting that an IAV-induced IFN response may be involved in causing increased expression of Bik.

Suppression of Bik expression caused reduction in the viral proteins M1 and M2. Such low viral protein production may result from a low progeny yield from the initial infection, which then causes reduced viral spread to other cells in the vicinity. Reduction in overall viral protein produced during infection with IAV was also observed in cells that overexpressed Bcl-2 (15) and in Bad-targeted cells (39). Similar observations were reported, in which blocking Bad prevented induction of cell death (39). During synthesis, the influenza A viral envelope proteins HA, NA, and M2 are transported to the ER for glycosylation and folding (40). BIK is integrated almost exclusively in the membrane of the ER (30, 11, 41) and binds to GRP78 (42), which keeps critical transmembrane ER-signaling proteins PERK, Ire1, and ATF6 in their inactive state (43). It is possible that, at least initially, Bik may be involved in the glycosylation and folding of IAV proteins. Thus, suppression of Bik level in the ER may inhibit glycosylation and proper folding of influenza A viral proteins thereby impairing viral replication.

Activation of the apoptotic pathway by IAV contributes to influenza virus pathogenesis, resulting in extensive lung tissue damage (44). Blockage of this cell death pathway leads to a significant decline in virus production (2). Our results showed that both *bik*$^{-/-}$ MAECs and HAECs with suppressed Bik expression were protected from IAV-induced cell death, suggesting that IAV caused cell death in a Bik- and caspase-dependent manner. Because Bik interacts and likely inactivates Bcl-2 (45), it is possible that Bik-deficient cells may stabilize or fail to properly inhibit Bcl-2 activity. Bik plays a significant role in IAV-induced caspase activation and efficient virus replication, because cleavage of caspase-3, as well as annexin V and PI positivity, were reduced in *bik* knockdown HAECs infected with IAV. Consistent with our findings, recent studies have shown Bik-dependent activation of caspases by the Epstein-Barr virus (20).

ER stress is involved in the pathogenesis of IAV (46), and BIK initiates ER Ca^{2+} release prior to the activation of effector caspases (11). In response to ER stress, GRP78 dissociates from the luminal domain of these sensors, leading to their activation (47). Thus, in addition to allowing the glycosylation and folding of IAV proteins, Bik may destabilize GRP78 to dissociate and activate IRE1, PERK, or ATF 6 to cause cell death and release of viral particles.

More interestingly, cleavages of viral NP and M2 proteins were inhibited in HAECs when Bik expression was suppressed and in cells in which caspase activation was blocked by the pan-caspase inhibitor (Q-VD). This supports a link between Bik-mediated caspase activation and cleavage of viral proteins, and it is reasonable to assume that Bik-mediated caspase cleavage of viral protein(s) promotes IAV replication. Activation of the apoptotic cascade plays a role in the processing of viral proteins and the maturation of viral particles (19). Several viruses, including IAV, express proteins that undergo host cell caspase cleavage (23). Influenza viruses are structured into ribo-NP segments consisting of viral RNA and viral proteins, the major one being the NP, a target of caspase cleavage (18). The viral ionic channel M2 protein is also cleaved by caspases (48, 49) in both human and avian influenza viruses. HIV-1 protease inhibitors are among the most effective antiretroviral drugs used successfully to prevent cleavage of HIV-1 viral proteins, resulting in superior antiviral activity (22). Thus, influenza A viral protein–activating host cell caspases may provide potential drug targets because of their role in virus infectivity and pathogenicity. Inhibition of host factors such as NP- or M2-activating caspases either by modulating the Bik level or by blocking the activity of specific caspases may be a novel approach to mitigating IAV replication. Future studies will identify and characterize specific caspase cleavage motifs of viral proteins and their specific role in Bik-mediated viral replication and pathogenesis.

Our immunofluorescence data showed increased nuclear accumulation and reduced cytoplasmic localization of viral NP when Bik was knocked down by shRNA. These observations suggest the potential for impaired shuttling of viral RNP to the cytoplasm by Bik deficiency. NPs or RNP have been shown to translocate partially to the cytoplasm after apoptotic stimuli in a caspase 3–dependent manner (16), and nuclear retention of NP caused by a lack of caspase activity has been linked to decreased titers of virus (14). Because IAV NP acts as a shuttle for viral genomic segments from the nucleus to the budding sites at the plasma membrane, localization during the virus replication cycle affects virus titers. Bcl-2 expression during infection leads to nuclear accumulation of IAV RNP complexes that causes improper assembly of progeny virions and a marked reduction in titers of infectious virus (14, 16, 18). It is not clear whether defects in the cleavage of viral proteins caused by Bik deficiency contribute partially to the nuclear retention of viral NPs. Caspase activation during apoptosis increases the permeability of the nuclear pores (50). Thus, IAV may use a strategy by which caspases regulate viral RNP export by increasing the diffusion limit of nuclear pores (50) to allow passive diffusion of viral RNP. There is potential that, in addition to cleaving viral proteins, Bik-mediated caspase activation may facilitate the transport of viral RNP to the cytoplasm as a complementary mechanism to allow the proper assembly of progeny virions.

Conclusions

In summary, we identified the proapoptotic BH3-only protein as a major factor in IAV replication. Bik deficiency is associated not only with diminished viral titer and reduced virus-induced cell death *in vitro*, but also with a significant increase in survival and a reduction in morbidity in influenza-infected mice. Bik promotes viral replication by mediating caspase 3–dependent cleavage of viral NP and M2 proteins and proper transport of viral RNP to the cytoplasm. Thus, Bik-mediated caspase cleavage of viral proteins and transport of viral RNP may play a significant role in the virus biology and pathogenicity linked with IAV replication. Therefore, inhibition of the proapoptotic host factor, Bik, or downstream effectors of cell death such as caspase 3, may represent a novel approach for treating the symptoms associated with influenza A infection. ■

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