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Ikkepsilon regulates viral-induced interferon regulatory factor-3 activation via a redox-sensitive pathway

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Abstract

Respiratory syncytial virus (RSV)-induced chemokine gene expression occurs through the activation of a subset of transcription factors, including Interferon Regulatory Factor (IRF)-3. In this study, we have investigated the signaling pathway leading to RSV-induced IRF-3 activation and whether it is mediated by intracellular reactive oxygen species (ROS) generation. Our results show that RSV infection induces expression and catalytic activity of IKKε, a noncanonical IKK-like kinase. Expression of a kinase-inactive IKKε blocks RSV-induced IRF-3 serine phosphorylation, nuclear translocation and DNA-binding, leading to inhibition of RANTES gene transcription, mRNA expression and protein synthesis. Treatment of alveolar epithelial cells with antioxidants or with NAD(P)H oxidase inhibitors abrogates RSV-induced chemokine secretion, IRF-3 phosphorylation and IKKε induction, indicating that ROS generation plays a fundamental role in the signaling pathway leading to IRF-3 activation, therefore, identifying a novel molecular target for the development of strategies aimed to modify the inflammatory response associated with RSV infection of the lung.

Keywords: RSV; Airway epithelial cells; IRF; ROS; Inflammation

Introduction

RSV is an enveloped, negative-sense single-stranded RNA virus, belonging to the paramyxovirus family. Since its isolation, RSV has been identified as a leading cause of epidemic respiratory tract illness in children, in the U.S. and worldwide (Hall, 2001). RSV induces a broad spectrum of clinical diseases ranging from otitis media to mild upper respiratory infection, acute laryngeo-tracheo-bronchitis, or more severe lower respiratory tract infections. In young children (1 month–2 years old), RSV infection can develop into significant lower respiratory tract disease, histologically distinguishable into two syndromes: (1) pneumonia, with diffuse mononuclear inflammation of the bronchi, bronchioles and interalveolar walls; and (2) bronchiolitis, characterized by necrosis of the bronchiolar epithelium, edema, and mononuclear cell infiltration of the bronchiolar submucosa with mucus plugs and air trapping (Aherne et al., 1970; Hall, 1999). Airway epithelial cells are the main targets of RSV infection. Following inhalation or self-inoculation of the virus into the nasal mucosa and infection of the local respiratory epithelium, RSV spreading along the respiratory tract occurs mainly by cell-to-cell transfer of the virus along the intracytoplasmic bridges (Hall, 1992). Under normal conditions, the respiratory epithelium is responsible for clearance of inhaled particulates and maintenance of alveolar patency. However,

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following inhalation of infectious agents, it is able to secrete a variety of molecules involved in antiviral and innate immune responses, like interferons, cytokines and chemokines, and therefore plays a major role in the initial host protective response to viral infections. A number of molecules have been described that are produced by human airway epithelial cells as a consequence of RSV infection. We, as well as others, have demonstrated that RSV is a potent stimulus for chemokine production, including interleukin (IL)-8 and RANTES, in cultured human nasal, bronchial and alveolar epithelial cells (Saito et al., 1997; Olszewska-Pazdrak et al., 1998; Zhang et al., 2001). In the past few years, we have characterized the mechanisms involved in RSV-induced expression of the chemokines IL-8 and RANTES, whose expression in epithelial cells require viral replication (Garofalo et al., 1996; Casola et al., 2000, 2001b). RSV-induced RANTES expression is dependent on the activation of IRFs, especially IRF-3, which are absolutely necessary for RANTES promoter induction (Casola et al., 2001b). We have also shown that ROS are important mediators of RSV-induced RANTES gene expression, modulating IRF-1, -3 and -7 activation (Casola et al., 2001a). Treatment of airway epithelial cells with the antioxidant butylated hydroxyanisol (BHA) inhibits RSV-induced IRF-1 and -7 gene expression and protein synthesis (Casola et al., 2001a), through inhibition of Signal Transducers and Activators of Transcription (STAT)-1 and -3 activation (Liu et al., 2004). BHA treatment also inhibits IRF-3 nuclear translocation, indicating that a redox-sensitive pathway is involved in RSV-induced IRF-3 activation (Casola et al., 2001a). However, the signaling pathway(s) leading to RSV-induced IRF-3 activation, and whether it is ROS-dependent, is currently not known.

IRF-3 is a constitutively expressed protein, which is phosphorylated on several serine/threonine residues present on the C-terminus of the protein, upon viral infection. This event allows dimerization, nuclear localization and DNA-binding (Lin et al., 1998). Two separate groups have identified the I kappa B kinase (IKK)-like molecule IKKε, together with Tank Binding Kinase (TBK)1, as a critical component of the virus-activated kinase (VAK) complex responsible for IRF-3 phosphorylation (Fitzgerald et al., 2003; Sharma et al., 2003). Overexpression of IKKε induces IRF-3 and -7 nuclear translocation and binding to Interferon Regulated Responsive Element (ISRE) promoter sites, leading to the expression IFN-β and RANTES genes (Fitzgerald et al., 2003; Sharma et al., 2003), following [(Fitzgerald et al., 2003; Sharma et al., 2003). In this study, we have investigated the role of IKKε, as well as ROS generation, in RSV-induced IRF-3 activation. RSV induces IKKε mRNA expression and protein synthesis, as well as its catalytic activity. Expression of a kinase-inactive IKKε blocks RSV-induced RANTES gene transcription, mRNA expression and protein synthesis. This occurs through inhibition of IRF-3 serine phosphorylation, an event known to be necessary for viral-induced nuclear translocation, DNA-binding and activation of chemokine gene transcription (Lin et al., 1998). Antioxidant treatment of alveolar epithelial cells inhibits RSV-induced IRF-3 serine phosphorylation and IKKε induction. Moreover, treatment of alveolar epithelial cells with various NAD(P)H oxidase inhibitors, including diphenylene iodonium (DPI), apocyanin and aminoethyl benzene sulfonyl fluoride (AEBSF), significantly reduces RANTES secretion, IRF-3 phosphorylation and IKKε induction, indicating that NAD(P)H oxidase-produced ROS are required for induction of the signaling pathway leading to IRF-3 activation in viral-infected airway epithelial cells.

Results

RSV infection of airway epithelial cells induces IKKε activation

IKKε is an IKK-like kinase identified as part of a novel kinase complex which can activate NF-κB following certain inducers like LPS and PMA (Shimada et al., 1999; Peters et al., 2000). Recent studies have reported a fundamental role of IKKε in IRF-3 activation and induction of IRF-3-dependent genes, like IFN-α4, IFN-β and RANTES (Sharma et al., 2003; Fitzgerald et al., 2003). Expression of IKKε can be either constitutive or inducible, depending on the cell type (Shimada et al., 1999). To investigate whether RSV infection of A549 cells induced IKKε expression, we performed Western blot analysis of total cell lysates prepared from A549 cells uninfected or infected for various lengths of time. As shown in Fig. 1A, RSV infection caused a marked increase in IKKε protein synthesis, starting around 6 h post-infection (p.i.) and peaking at 12 h. To determine whether IKKε protein synthesis was paralleled by changes in steady-state level of its mRNA, total RNA was extracted from cells uninfected and infected for various length of time, and used to amplify IKKε gene by RT-PCR (Olszewska-Pazdrak et al., 1998). As shown in Fig. 1B, RSV-induced IKKε gene expression followed a kinetic similar to the one seen for protein induction, with up-regulation occurring between 6 and 12 h p.i.

We then asked whether RSV-induced IKKε was catalytically active and could phosphorylate IRF-3. For this purpose, we performed kinase assays using IKKε immunoprecipitated from A549 cell uninfected or infected with RSV for various lengths of time and an IRF-3 peptide corresponding to amino acids 380–406, which contains the major viral-inducible phosphorylation sites (Lin et al., 1998), as substrate. As shown in Fig. 1C, IKKε catalytic activity was significantly increased, following viral infection, starting at 6 h p.i., with maximal activity at 12 h. This pattern is similar to the kinetics of RSV-induced IRF-3 nuclear translocation and DNA binding, as well as to the kinetics of RSV-induced RANTES gene expression (Casola et al., 2001a).

Viral-induced IRF-3 activation occurs through phosphorylation of specific C-terminal serine residues, located between amino acids 386 and 405 (Lin et al., 1998), of which Ser396 has been shown to be necessary for viral-induced IRF-3 activation (Servant et al., 2003). To determine whether IRF-3 was phosphorylated following RSV infection, cytoplasmic and nuclear extracts were prepared from A549 cells uninfected or infected for various length of time and used for Western blot analysis of Ser396 phosphorylated IRF-3. As shown in Fig. 1D, RSV infection induced IRF-3 phosphorylation both in the cytoplasmic and nuclear compartment, starting at 6 h p.i. and

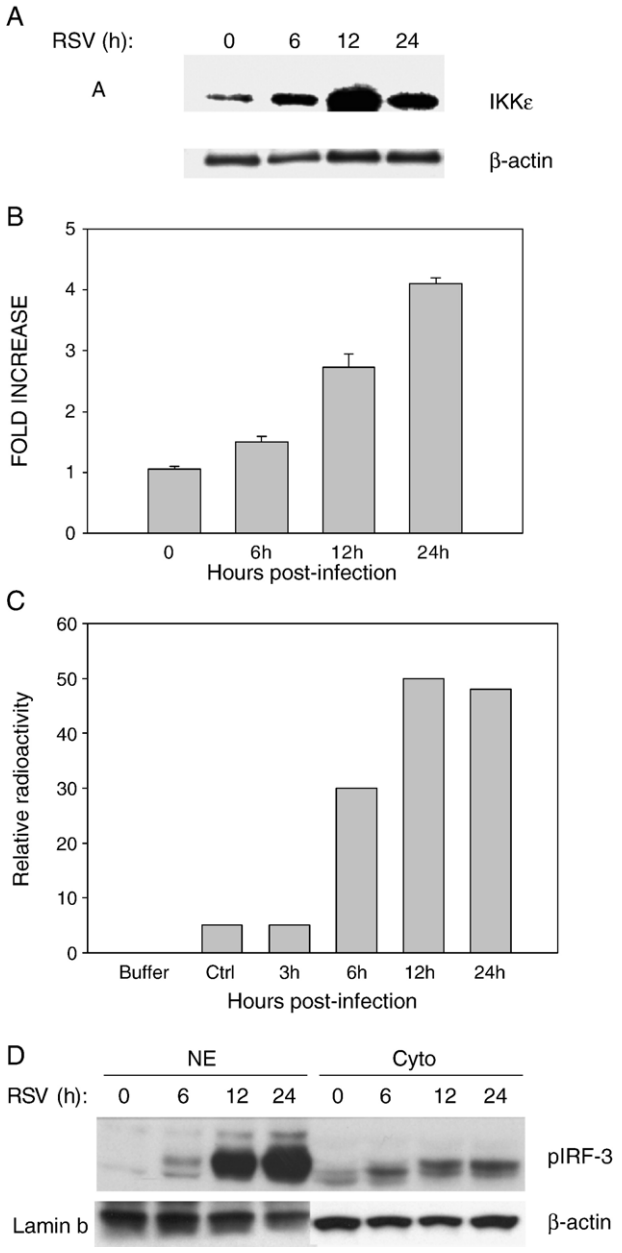

Fig. 1. RSV infection induces IKKε activation in A549 cells. Panel A: IKKε protein levels. Total cell lysates, prepared from A549 cells uninfected or infected with RSV for 6, 12 and 24 h, were resolved on 10% SDS-PAGE and Western blot was performed using an anti-IKKε antibody. Membrane was stripped and reprobed for β-actin. Panel B: IKKε mRNA. A549 cells were infected with RSV for various lengths of time (hours). Total RNA was extracted from control and infected cells and used to amplify IKKε by Q-RT-PCR. Panel C: IKKε kinase activity. Total cell lysates were prepared from uninfected (Ctrl) or RSV-infected cells for 3, 6, 12 and 24 h. IKKε was immunoprecipitated with a specific antibody for in vitro kinase assay. Amount of ^{32}P-phosphate incorporated into the IRF-3 substrate was quantified by exposure to PhosphoImager cassette and analyzed with Molecular Dynamics Storm imaging system software. Relative radioactivity is expressed in arbitrary units after subtraction of the background radioactivity present in the buffer alone sample. Data are representative of one of two independent experiments. Panel D: kinetics of IRF-3 phosphorylation. A549 cells were infected with RSV for various lengths of time and harvested to prepare cytoplasmic (Cyto) and nuclear extracts (NE). Equal amounts of protein from uninfected and infected cells were analyzed by Western immunoblot probed with anti Ser396 phospho-IRF-3 antibody. Membrane was stripped and reprobed for β-actin (cytoplasmic extracts) and lamin b (nuclear extracts).

peaking at 24 h p.i,. The kinetics of phospho-Ser396 IRF-3 formation is similar to RSV-induced IKKε activation, suggesting that indeed IKKε could play an important role in IRF-3 activation following RSV infection.

IKKε regulates RSV-induced RANTES promoter activation through the ISRE site

Since IRF-3 activation plays a fundamental role in RSV-induced chemokine gene expression (Casola et al., 2001b), we investigated the effect of overexpressing catalytically inactive IKKε (mutated on the lysine residue 38 of the catalytic domain by substitution with alanine and defined as K38A) (Peters et al., 2000) on RSV-induced RANTES gene transcription. 293 cells were cotransfected with a construct containing the minimal RSV-inducible RANTES promoter-luciferase reporter gene, PGL2-220, and a pcDNA3-based expression plasmid containing FLAG-tagged IKKε K38A (Casola et al., 2001b). Expression of the dominant negative mutant IKKε significantly reduced, in a dose-dependent manner, RSV-induced luciferase activity of the RANTES promoter construct, indicating a role of this kinase in viral-induced RANTES gene transcription (Fig. 2A).

RANTES promoter activation, following RSV infection, is controlled by multiple regulatory elements (Casola et al., 2001b). Both an intact NF-κB site and ISRE, the IRF binding site, are important for RSV-stimulated RANTES gene transcription, with the ISRE site being absolutely necessary (Casola et al., 2001b). To determine whether IKKε activation was sufficient to induce RANTES transcription and whether this occurred through the ISRE site, 293 cells were transiently transfected with a plasmid expressing wild type IKKε and RANTES promoter plasmids either wild type or containing site-mutations of the ISRE or the NF-κB binding sites. As shown in Fig. 2B, overexpression of IKKε caused a significant (~6-fold) activation of the RANTES promoter, which was abolished by mutation of the ISRE site. On the contrary, the RANTES NF-κB site mutation significantly reduced basal promoter activity, yet showed a 20-fold induction by IKKε expression (Casola et al., 2001b). Together these data indicate that IKKε is a potent activator of the ISRE.

To further confirm that expression of the catalytically inactive IKKε affected RSV-induced RANTES promoter activation through the ISRE site, 293 cells were transiently transfected with a construct containing multiple copies of the isolated RANTES ISRE site linked to the luciferase reporter gene (Casola et al., 2002), and the IKKε K38A plasmid. Expression of IKKε K38A caused a significant inhibition, between 70 and 80%, of IRF-driven transcription (Fig. 2C). This effect was specific to IKKε since expression of catalytically inactive (DN) IKKβ only modestly reduced RSV-induced ISRE activation (Fig. 2C).

IKKε regulates RSV-induced RANTES gene expression and IRF-3 phosphorylation

To further investigate the role of IKKε in transcription factor activation and gene expression following RSV infection, we

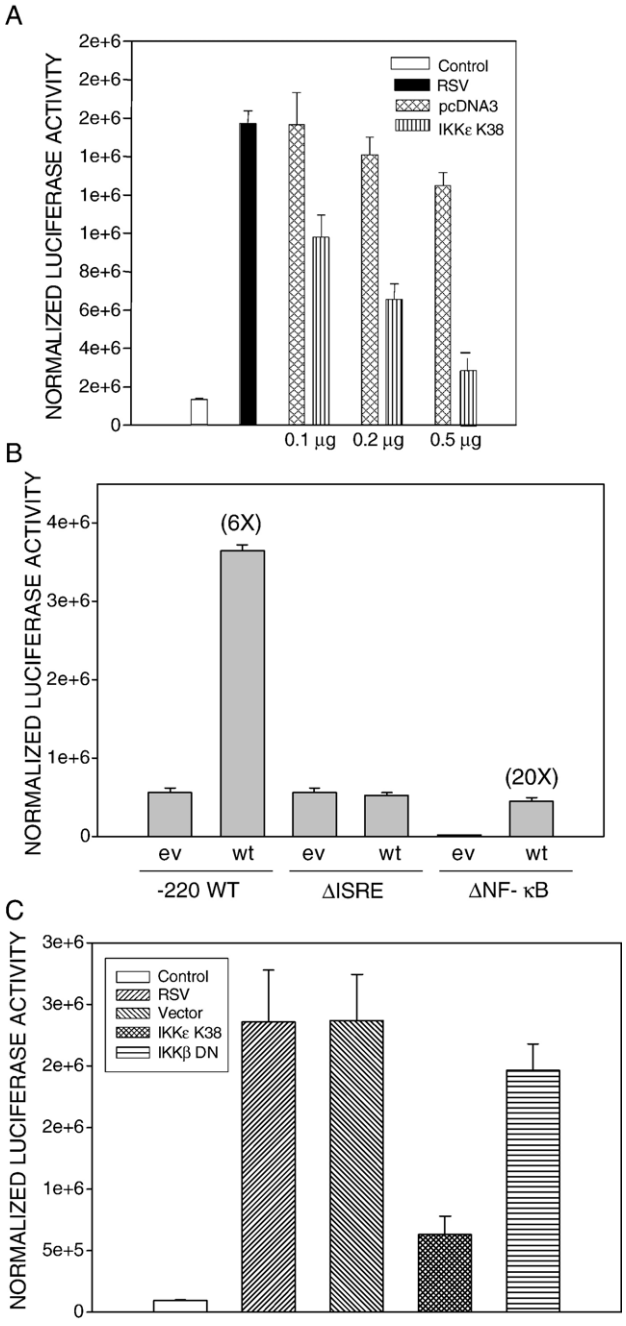

Fig. 2. Effect of overexpressing catalytically-inactive or wild type IKKε on RANTES transcription. Panel A: 293 cells were transfected with the PGL2-220 RANTES promoter and the pcDNA3-IKKε K38A plasmid or the empty vector at different indicated concentrations, and infected with RSV. Cells were harvested 24 h post-infection to measure luciferase activity. Uninfected plates served as controls. For each plate luciferase was normalized to the β-galactosidase reporter activity. Data are expressed as mean ± standard deviation of normalized luciferase activity. Panel B: 293 cells were transfected with the PGL2-220 RANTES promoter wild type (WT) or mutated in the ISRE (ΔISRE) or NF-κB (ΔNF-κB) site and the pcDNA3-IKKε wild type (WT) or the empty vector (EV). Cells were harvested 48 h later to measure luciferase activity. Panel C: 293 cells were transiently transfected with multimers of the RANTES ISRE site and either the pcDNA3-IKKε K38A plasmid or a kinase-inactive (DN) IKKβ expression plasmid and infected with RSV. Cells were harvested 12 h post-infection to measure luciferase activity. Uninfected plates served as controls. For each plate luciferase was normalized to the β-galactosidase reporter activity. Data are expressed as mean ± standard deviation of normalized luciferase activity.

established 293 stable cell lines expressing K38A FLAG-tagged IKKε, under the control of a tetracycline-regulated promoter. As a control cell line, we used a clone containing the empty vector (EV) only. To induce IKKε expression, the stable tetracycline derivative doxycycline (Dox) was added to the culture medium and IKKε protein level was monitored at different time points thereafter by Western blot using an anti-FLAG antibody. As shown in Fig. 3, there was a time-dependent expression of IKKε, which was maximal at 48 h following Dox treatment (further time points not shown). We next sought to confirm the role of IKKε in RSV-induced RANTES gene expression by investigating RANTES protein and mRNA induction following RSV infection of the K38A-expressing 293 cell line. Cells were treated with Dox for 48 h, infected with RSV for 24 h and harvested to measure RANTES protein by ELISA and RANTES mRNA by Northern blot (Casola et al., 2001a). Expression of kinase-inactive IKKε significantly blocked viral-induced RANTES secretion (Fig. 4A), as well as mRNA induction (Fig. 4B), indicating a fundamental role of IKKε in the expression of this viral-induced chemokine. A similar inhibition was observed for RSV-induced production of IFN-β, whose gene expression is regulated similarly to the RANTES gene (data not shown) (Wathelet et al., 1998).

Furthermore, to determine whether inhibition of IKKε activation would block RSV-induced IRF-3 activation, we measured phospho Ser396 IRF-3 formation in 293 cells expressing IKKε K38A. Nuclear extracts were prepared from control (EV) or IKKε K38A (DN) expressing 293 cells infected with RSV for various lengths of time and used for Western blot analysis of phospho Ser396 IRF-3. As shown in Fig. 4C, expression of catalytic-inactive IKKε significantly blocked viral-induced IRF-3 phosphorylation, indicating that IKKε plays a fundamental role in RSV-induced IRF activation.

ROS regulate viral-induced IRF-3 activation

We have previously shown that RSV infected airway epithelial cells generate ROS and that antioxidant treatment with butylated hydroxyanisole (BHA), as well as a panel of chemically unrelated antioxidants, blocks RSV-induced signal transduction cascade leading to chemokine expression in vitro, through inhibition of transcription factors belonging to IRF and Signal Transducers and Activators of Transcription (STAT)

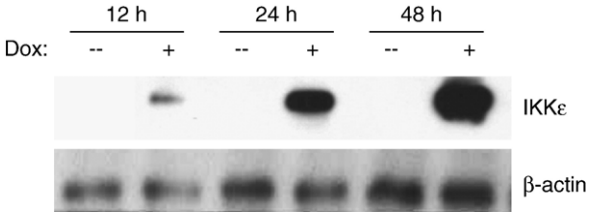

Fig. 3. Kinetics of catalytically inactive IKKε expression in 293 stable cell line. 293 stable cell lines IKKε K38A were treated with Dox for 12, 24 and 48 h and harvested to prepare total cell lysates. Equal amounts of protein from uninfected and infected cells were assayed by Western blot for FLAG-IKKε expression. Membrane was stripped and reprobed for β-actin to control the equal loading of the samples.

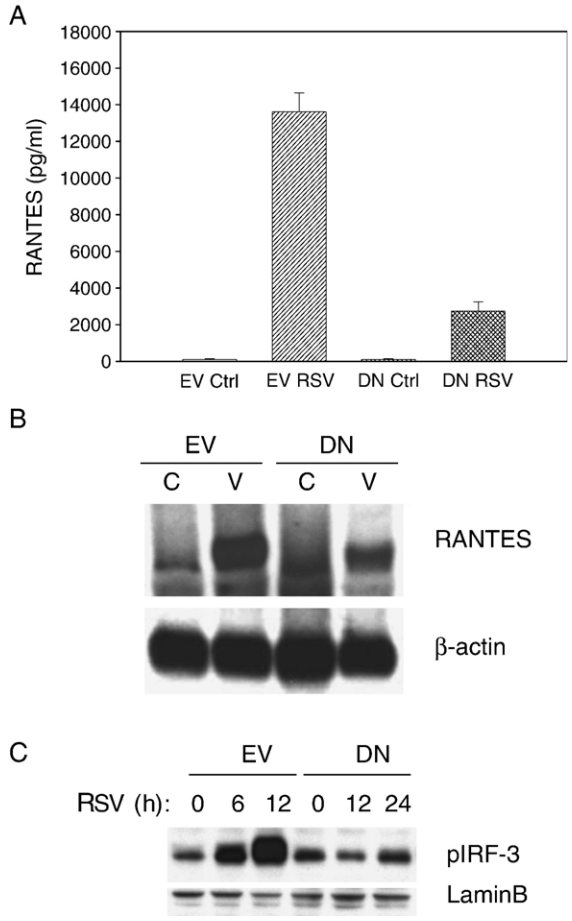

Fig. 4. Expression of catalytically inactive IKKε blocks viral-induced RANTES expression and IRF-3 phosphorylation. Panel A: RANTES protein secretion. 293 control cell line (EV) and IKKε K38A cell line (DN) were treated with tetracycline for 48 h and then infected with RSV. Culture supernatants, from uninfected (Ctrl) and infected (RSV) cells, were assayed 24 h later for RANTES production by ELISA. Data are expressed as mean ± standard deviation of triplicate samples. Panel B: RANTES mRNA induction. Total RNA was extracted from control (C) and 24 h infected (V) cells. Twenty micrograms of RNA were fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membrane and hybridized to a radiolabeled RANTES cDNA probe. Membrane was stripped and hybridized with a radiolabeled β-actin probe, to show equal loading of the samples. Panel C: IRF-3 phosphorylation. Control and IKKε K38A 293 cell lines were infected with RSV for 6, 12 and 24 h and harvested to prepare nuclear extracts. Equal amounts of protein from uninfected and infected cells were fractionated on a 10% SDS-PAGE, transferred to PVDF membrane and probed with anti Ser396 phospho-IRF-3 antibody. Membrane was stripped and reprobed for lamin b to control the equal loading of the samples.

families (Casola et al., 2001a; Liu et al., 2004). BHA treatment blocks RSV-induced IRF-1 and -7 gene expression and inhibits IRF-3 nuclear translocation and DNA binding to the RANTES Interferon Stimulated Responsive Element (ISRE), an event that is absolutely required for RSV-stimulated RANTES gene transcription. To determine whether antioxidant treatment inhibits RSV-induced IRF-3 activation, we examined RSV-induced IRF-3 serine phosphorylation in A549 cells treated with 400 μM BHA. A549 cells were infected with RSV in the presence or absence of BHA and harvested at 12 and 24 h p.i. to extract nuclear proteins for Western blot analysis of phosphor Ser396 IRF-3. As shown in Fig. 5A, antioxidant treatment completely blocked RSV-induced IRF-3 phosphorylation, indicating that it is redox-dependent.

Since IKKε plays a major role in RSV-induced IRF-3 activation, we examined IKKε gene expression and protein synthesis in A549 cells infected with RSV in the presence or absence of the antioxidant. As shown in Fig. 5B, RSV-induced IKKε gene expression was significantly inhibited by antioxidant treatment. Similar results were obtained when we examined IKKε protein synthesis, which was strongly induced by RSV infection and abolished by antioxidant treatment (Fig. 5C).

An important source of inducible intracellular ROS, generated in response to a variety of stimuli, is the membrane-bound NAD(P)H oxidase system [Reviewed in (Babior, 1999)]. We have previously shown that treatment of A549 cells with DPI, a known NAD(P)H oxidase inhibitor, significantly

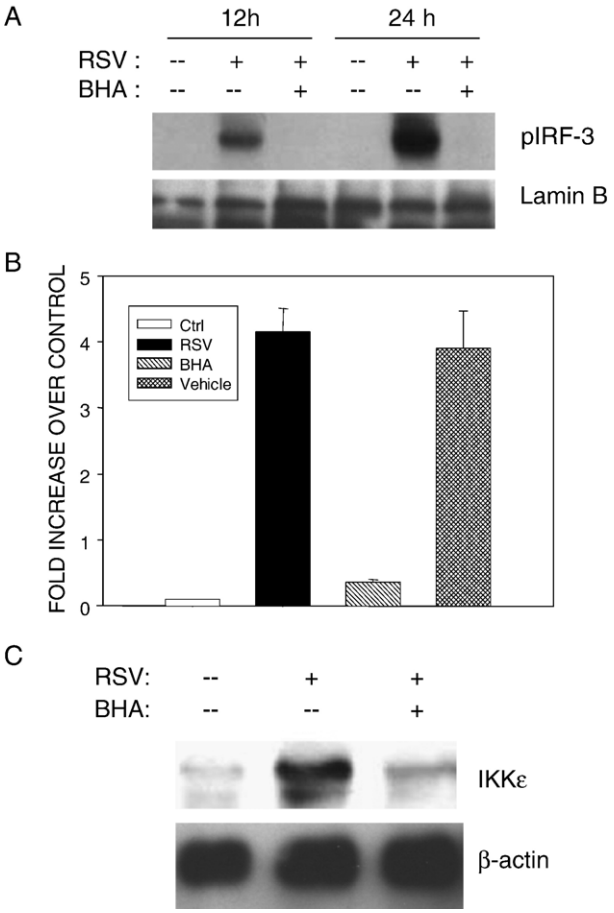

Fig. 5. Effect of antioxidant treatment on RSV-induced IRF-3 and IKKε activation. Panel A: IRF-3 phosphorylation. Nuclear extracts were prepared from A549 cells control and infected with RSV for 12 and 24 h, in the absence or presence of 400 μM BHA, and assayed for Ser396 phosphorylation by Western blot. Membrane was stripped and reprobed for lamin b to control the equal loading of the samples. Panel B: IKKε mRNA. A549 cells were infected with RSV for 24 h, in the absence or presence of 400 μM BHA. Total RNA was extracted from control and infected cells and used to amplify IKKε by Q-RT-PCR. Panel C: IKKε protein levels. Total cell lysates, prepared from A549 cells control and infected with RSV for 24 h, in the absence or presence of 400 μM BHA, were resolved on 10% SDS-PAGE and Western blot was performed using an anti-IKKε antibody. Membrane was stripped and reprobed for β-actin.

blocked RSV-induced STAT activation (Liu et al., 2004). To test if RSV-induced IRF-3 activation was mediated through ROS production by this enzyme complex, A549 cells were treated with various NAD(P)H oxidase inhibitors, including DPI, apocyanin and AEBSF (Schwarzer et al., 2004), and infected with RSV for 24 h. We then assessed RSV-induced RANTES production and IRF-3 phosphorylation. As shown in Fig. 6A, all three inhibitors significantly reduced RANTES secretion, as well as IRF-3 phosphorylation (Fig. 6B). DPI almost completely abolished RSV-induced IKKε gene expression (Fig. 6C), with a limited effect on viral replication (Fig. 6D), indicating that the NAD(P)H oxydase system is important for the generation of ROS and subsequent activation of IRF-3 following RSV infection.

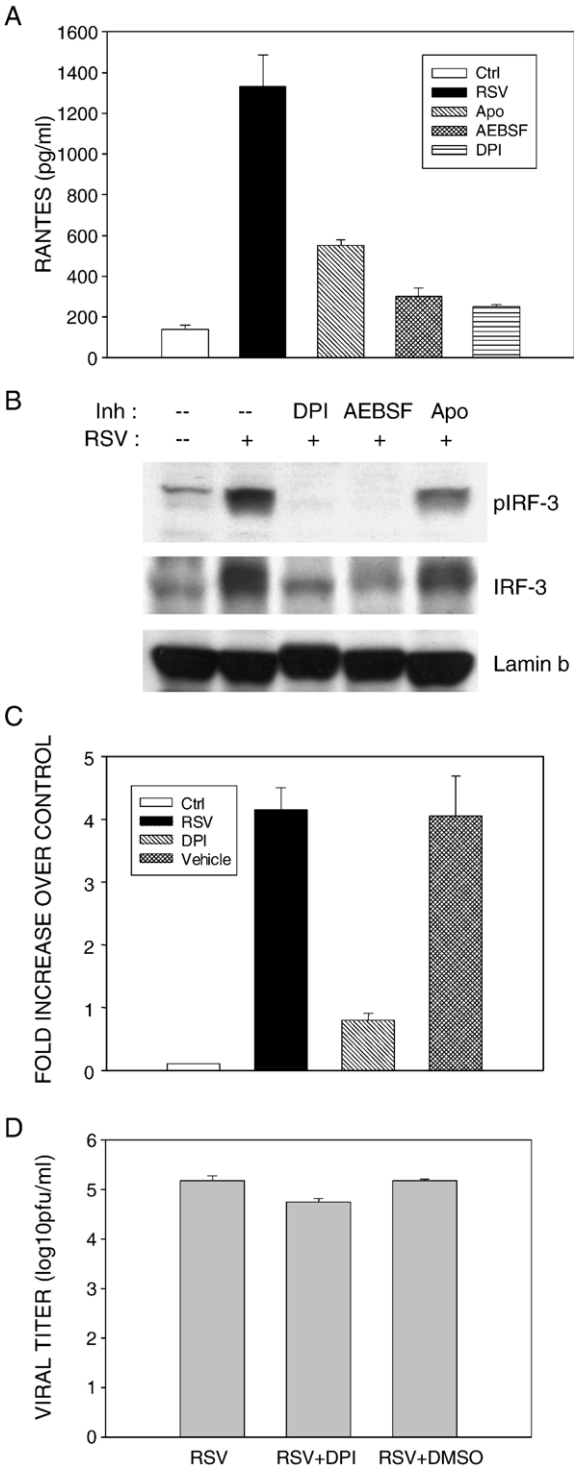

Fig. 6. Effect of NADPH inhibition on RSV-induced IRF-3 and IKKε activation. Panel A: RANTES secretion. A549 cells were infected with RSV for 24 h, in the absence or presence of either 10 μM DPI, 500 μM AEBSF or 500 μM apocyanin (Apo). Culture supernatants were assayed 24 h later for RANTES production by ELISA. Data are expressed as mean ± standard deviation of triplicate samples. Panel B: IRF-3 phosphorylation. Nuclear extracts were prepared from A549 cells control and infected with RSV for 24 h, in the absence or presence of either 10 μM DPI, 500 μM AEBSF or 500 μM apocyanin (Apo) and assayed for Ser396 phosphorylation by Western blot. Membrane was stripped and reprobed for total IRF-3 and for lamin b to control the equal loading of the samples. Inh: inhibitor. Panel C: IKKε mRNA. A549 cells were infected with RSV for 24 h, in the absence or presence of 10 μM DPI. Total RNA was extracted from control and infected cells and used to amplify IKKε by Q-RT-PCR. Panel D: viral replication. A549 cells were infected with RSV, in the absence or presence of 10 μM DPI. Cell culture supernatants were harvested at 24 h post-infection and viral load was determined by plaque assay.

Discussion

The innate immune response represents a critical component of the host defense against viruses and is coordinated at the cellular level by activation of transcription factors that regulate the expression of inducible gene products with antiviral and/or inflammatory activity. RSV is the most common cause of epidemic respiratory infections in infants and young children and represents the prototype of mucosal-restricted viral pathogens with epithelial cell tropism and profound proinflammatory activity. RSV replication in airway epithelial cells results in the activation of multiple cellular signaling pathways involved in the expression of early response genes, which is coordinated by a small subset of transcription factors including NF-κB and IRF proteins (Garofalo et al., 1996; Casola et al., 2000, 2001b; Tian et al., 2002). Most of the signaling pathways leading to RSV-induced gene expression are still unknown. Free radicals and ROS have recently been shown to function as second messengers influencing a variety of molecular and biochemical processes, including expression of a number of genes [Reviewed in (Allen and Tresini, 2000)]. In earlier studies, we have shown that ROS play a major role in RSV-induced de novo IRF-1 and -7 genes expression and protein synthesis, by regulating STAT-1 and -3 activation (Casola et al., 2001a; Liu et al., 2004). We have also shown that inhibition of ROS production, by antioxidant treatment, blocks IRF-3 nuclear translocation and DNA binding to the RANTES ISRE site (Casola et al., 2001a). However, the intracellular signaling events leading to RSV-induced IRF-3 activation and the mechanism of inhibition of IRF-3 induction by antioxidant treatment were not investigated. In this study, we show that RSV infection of airway epithelial cells induces activation of IKKε, a noncanonical IKK-like molecule, recently identified as an essential component of the virus activated kinase (VAK) complex responsible for IRF-3 activation. Expression of catalytically inactive IKKε significantly inhibits RSV-induced

RANTES expression, by blocking RANTES promoter activation and ISRE-driven transcription, through the inhibition of IRF-3 phosphorylation, indicating a fundamental role of this kinase in the pathway leading to RSV-induced IRF-3 activation. We also show that RSV-induced ROS formation, occurring likely through the NAD(P)H oxidase system, is involved in RSV-induced IKKε activation, as indicated by the inhibition of IKKε expression and IRF-3 phosphorylation by antioxidant treatment, as suggested in the schematic model of Fig. 7.

IKKε was originally cloned as a component of a distinct IκB kinase complex, separate from the canonical one containing IKKα and IKKβ, that mediates NF-κB activation following certain inducers like LPS and phorbol ester (Shimada et al., 1999; Peters et al., 2000). However, recent findings have revealed a fundamental role of IKKε, and the similar IKK-like kinase Tank Binding Kinase (TBK)1, in the signaling pathway leading to IRF-3 activation (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; Perry et al., 2004). Both kinases have been shown to be important for double-stranded RNA and viral-induced IRF-3 phosphorylation and expression of IRF-3 dependent genes, while TBK1, but not IKKε, seems to be required for LPS-induced IRF-3 activation (Hemmi et al., 2004; Perry et al., 2004; TenOever et al., 2004).

While TBK1 is constitutively and ubiquitously expressed, expression of IKKε can be induced, in a cell type-dependent manner (Shimada et al., 1999). Splenocytes and thymocytes show constitutive expression of IKKε, while IKKε mRNA can be upregulated in macrophages and cells isolated from the synovia following cytokines, LPS or PMA stimulation (Shimada et al., 1999; Aupperle et al., 2001). Here, we show that IKKε expression is inducible in airway epithelial cells infected with RSV. The only another report of IKKε induction following a viral infection, is in mouse embryonic fibroblasts infected with Vesicular Stomatitis Virus (VSV) (TenOever et al., 2004). The mechanism(s) regulating IKKε gene expression is not known, however cytokines, LPS, PMA and viruses are all potent activators of NF-κB, therefore it is likely that this master transcriptional regulator plays an important role in IKKε induction. In support of this hypothesis is the lack of IKKε gene expression in LPS-stimulated MEFs which are deficient in the expression of IKKβ, the kinase that controls NF-κB activation following a variety of different stimuli, including viral infections (Chu et al., 1999; Delhase et al., 1999; Israel, 2000).

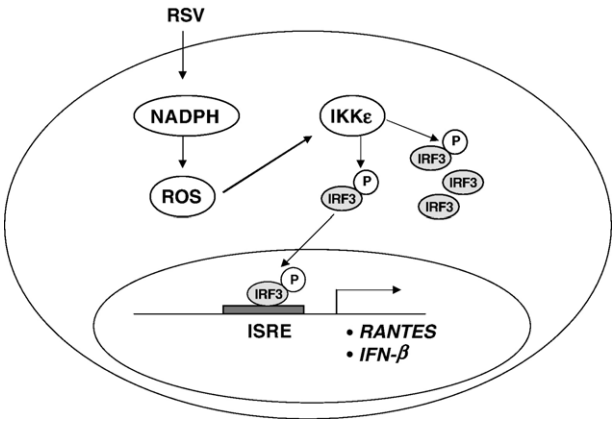

Fig. 7. Schematic representation of the proposed role of ROS in viral-induced IKKε and IRF-3 activation.

In our study, the kinetics of IKKε kinase activity correlates with its expression. It has been previously shown that recombinant IKKε is phosphorylated during its expression and that this phosphorylation is necessary for kinase activity (Shimada et al., 1999; Kishore et al., 2002). Once the kinase is present, its enzymatic activity cannot be further stimulated by agonist treatment, suggesting that IKKε activity is likely regulated at the level of mRNA induction and not at the level of phosphorylation by stimuli-activated kinases (Shimada et al., 1999; Kishore et al., 2002).

Phosphorylation is a fundamental mechanism of IRF protein activation, necessary for nuclear translocation, dimerization, binding to DNA and activation of transcription [Reviewed in (Servant et al., 2001)]. Upon viral infection, both IRF-3 and IRF-7 are phosphorylated within the C terminus of the protein (Mamane et al., 1999). Although recent studies indicated that IRF-3 may also be a phosphorylation target following stimulation of cellular stress pathways or the engagement of TLR receptors like TRL3 and 4 [Reviewed in (Servant et al., 2002)], only Sendai Virus, Measles Virus, Newcastle Disease Virus, VSV and RSV have been clearly shown to induce C-terminal phosphorylation of IRF-3 (Servant et al., 2001; Casola et al., 2001a; tenOever et al., 2002). Among the major viral-inducible phosphoacceptor sites present in the C-terminal portion of IRF-3, Ser396 has been shown to be the minimal phosphoacceptor site involved in IRF-3 activation following infection with Sendai virus and double-stranded RNA treatment (Servant et al., 2003). Using a novel phosphospecific antibody (Servant et al., 2003), we show that RSV infection induces Ser396 phosphorylation, which is completely abolished by the expression of catalytically inactive IKKε, indicating a fundamental role of this kinase in RSV-induced IRF-3 activation. The evidence that IKKε, following RSV infection, is able to phosphorylate a C-terminal IRF-3 peptide, containing the major viral-inducible phosphoacceptor sites, with a kinetics very similar to the kinetics of RSV-induced IRF-3 activation (Casola et al., 2001a) strongly suggests that IKKε is a component of the virus-activated kinase complex required for IRF-3 phosphorylation following RSV infection. Since TBK1 has also been shown to play an important role in viral-induced IRF-3 activation, we are currently investigating the contribution of this kinase in IRF-3 activation following RSV infection.

In this study, we show that ROS formation plays a major role in the signaling pathway leading to viral-induced IRF-3 activation. Antioxidant treatment blocks IKKε expression and subsequent IRF-3 phosphorylation, following RSV infection, identifying this pathway as redox-sensitive. This is a novel observation, since it is the first time that viral-induced ROS production are shown to be involved in IKKε activation and subsequent IRF-3 phosphorylation. NF-κB could be the redox-sensitive target involved in IKKε gene transcription, since ROS

have been shown to play an important role in NF-κB activation following various stimuli (Schieven et al., 1993; Schoonbroodt and Piette, 2000). Intracellular ROS can be generated by different systems, including the NAD(P)H oxidase system, the mitochondrial electron transport chain and enzymes like xanthine oxidase and cyclooxygenase. ROS play an important role in the regulation of intracellular signaling cascades in various cell types including fibroblasts, endothelial cells, myocytes, smooth muscle cells, etc. [Reviewed in (Droge, 2002)]. In our study, treatment of RSV-infected alveolar epithelial cells with a panel of inhibitors of the NAD(P)H complex significantly reduced RANTES production and IRF-3 activation. DPI also almost completely abolishes viral-induced IKKε gene expression. These data suggest that NAD(P)H oxidase is an important system for the generation of ROS and subsequent activation of IRF-3, following RSV infection. Since inhibitors may not be completely specific for NAD(P)H oxidase, current studies are in progress to better define the role of this complex in RSV-induced ROS formation and IRF-3 activation. However, the recent finding that Rac1, a small GTPase part of the NAD(P)H complex , is necessary for influenza virus-induced activation of IRF-3 and IRF-3-dependent genes (Ehrhardt et al., 2004), supports the role of NAD(P)H-generated ROS as fundamental mediators of the viral-induced signaling pathway leading to IRF-3 activation.

In summary, RSV-induced chemokine gene expression is controlled by numerous ROS-sensitive signaling molecules. This study indicates that viral-induced ROS formation, occurring likely through the NAD(P)H oxidase-generated system, is necessary for IRF-3 phosphorylation, through modulation of IKKε activation, and identifies a novel molecular target for the development of strategies aimed to modulate the inflammatory response associated with RSV infection of the lung.

Materials and methods

RSV preparation

The RSV A2 strain was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (Ueba, 1978). The virus titer of the purified RSV pools, was 8–9 $\log_{10}$ plaque forming units (PFU)/ml using a methylcellulose plaque assay. No contaminating cytokines were found in these sucrose-purified viral preparations (Patel et al., 1995). LPS, assayed using the limulus hemocyanin agglutination assay, was not detected. Virus pools were aliquoted, quick-frozen on dry ice/alcohol and stored at −70 °C until used.

Cell culture and infection of epithelial cells with RSV

A549, human alveolar type II-like epithelial cells, and 293, a human embryonic kidney epithelial cell line (both from ATCC, Manassas, VA), were maintained in F12K and MEM medium respectively, containing 10% (v/v) FBS, 10 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell monolayers were infected with RSV at multiplicity of infection (MOI) of 3 (unless otherwise stated), as described (Garofalo et al., 1996). An equivalent amount of a 30% sucrose solution was added to uninfected A549 cells, as a control. When inhibitors were used, cells were pretreated for 1 h and then infected in the presence of the compound. Since inhibitors were diluted either in ethanol or in DMSO, equal amount of diluent was added to untreated cells, as a control. Total number of cells and cell viability, following treatment, were measured by trypan blue exclusion. No cell toxicity was observed for any compound at concentrations used for the experiments.

Western blotting

Total cell lysates were prepared by adding ice-cold lysis buffer (50 mM Tric–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1% Triton X-100 and 1 μg/ml of aprotinin, leupeptin and pepstatin. After incubation on ice for 10 min, the lysates were collected and detergent insoluble materials were removed by centrifugation at 4 °C at 14,000 × *g*. Cytoplasmic and nuclear extracts were prepared using hypotonic/nonionic detergent lysis, as previously described (Casola et al., 2000). Proteins (20 to 50 μg per sample) were then boiled in 2× Laemmli buffer for 2 min and resolved on SDS-PAGE. Proteins were transferred for 6 h onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech) and nonspecific binding sites were blocked by immersing the membrane in TBST blocking solution [10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 (v/v)] containing 5% skim milk powder or 5% bovine serum albumin for 30 min. After a short wash in TBST, the membranes were incubated with the primary antibody overnight at 4 °C, followed by an anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), diluted 1:10,000 in TBST for 30 min at room temperature. After washing, the proteins were detected using ECL (Amersham Pharmacia Biotech) according to manufacturer's protocol. The primary antibodies used for Western blots were: rabbit anti-IKKε polyclonal (Millennium Pharmaceutical), rabbit polyclonal antibody to phospho-IRF-3, which recognize the viral-induced C-terminal phosphorylated form of the molecule (Servant et al., 2003), (a gift from Rongtuan Lin, McGill University, Montreal, Canada), rabbit polyclonal antibodies against IRF-3 (Santa Cruz).

RANTES ELISA

Immunoreactive RANTES was quantified by a double antibody ELISA kit (DuoSet, R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

Northern blot and real time PCR

Total RNA was extracted from control and infected A549 cells by the acid guanidium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987). Twenty micrograms of RNA were fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membranes and hybridized to a radiolabeled RANTES cDNA, as previously described (Casola et al.,

2001b). After washing, membranes were exposed for autoradiography using Kodak XAR film at −70 °C, using intensifying screens. After exposure, membranes were stripped and rehybridized with a β-actin probe. For IKKε amplification by real time PCR, Quantitative Real Time PCR (Q-RT-PCR) Applied Biosystems assays-on-demand 20× mix of primers and TaqMan MGB probes (FAM-dye labeled) for target genes and 18S rRNA (VIC-dye labeled probe) TaqMan assay reagent (P/N 4319413E) for controls were used. Separate tubes (singleplex) one-step RT-PCR was performed with 80 ng RNA for both target genes and endogenous control. The cycling parameters for one-step RT-PCR was: reverse transcription 48 °C for 30 min, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s and annealing/extension 60 °C for 1 min (repeat 30 times) on ABI7000. Duplicate CT values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to endogenous reference (18S) sample.

Plasmid construction and cell transfection

The PGL2-220 plasmid, containing the first 220 nucleotides of the RANTES promoter, the IL-8 LUC, containing the first −162 nucleotides of the IL-8 promoter, as well as the plasmid containing multiple copies of the RANTES ISRE site linked to the luciferase reporter gene, have been previously described (Casola et al., 2000, 2001b, 2002). The IKKε wild type and catalytically-inactive (K38A) plasmids were created by cloning the PCR product corresponding to bases 324 to 2477 of the human IKKε gene into the pcDNA3 expression vector, using the *Bam*HI and *Xba*I cloning sites, as previously described (Peters et al., 2000). Mutation of the lysine 38 residue to alanine was introduced by overlapping PCR (Peters et al., 2000). The plasmid expressing a kinase inactive (DN) IKKβ was a generous gift of Dr. Mercurio, Signal Pharmaceutics, and has been previously described (Mercurio et al., 1997).

Logarithmically growing A549 or 293 cells were transfected in triplicate in 60 mm dishes using Fugene 6 (Roche, Indianapolis, Ind.). One μg of the reporter gene plasmid, 0.5 μg of β-galactosidase and 0.5 μg of either IKKε wild type or K38A or IKKβ DN expression plasmid were premixed with FuGene6 in a 1:3 ratio (μg/μl), and added to the cells in 3 ml of regular medium. The next morning, cells were infected with RSV and at 12 or 24 h post-infection cells were lysed to measure independently luciferase and β-galactosidase reporter activity, as previously described (Casola et al., 2000). Luciferase was normalized to the internal control β-galactosidase activity. All experiments were performed at least two to three times.

IKKε stable cell lines

Tetracycline-inducible 293-IKKε wild type and kinase inactive cell lines were generated using a modified version of the Tet-on system (Yamamoto et al., 2001). 293T cells were transfected with plasmids encoding a reverse tetracycline-inducible transactivator, a tetracycline-regulated repressor, the puromycin resistance gene and IKKε (wild type or K38A mutant) and GFP under the control of a bi-directional promoter (plasmids were kindly provided by Dr. Jay Morgenstern, Millennium Pharmaceuticals). Transfection was performed using Fugene 6, according to the manufacturer's instructions. Stably transfected cell lines were selected in the presence of puromycin. Flow cytometry sorting was used to select cells that were GFP-negative in the absence of doxycycline and GFP-positive in its presence. Cells were sorted as single cells into 96-well plates and resulting clones were tested for inducible IKKε protein expression by Western blot analysis.

Kinase assays

A549 cells were resuspended in lysis buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 10.0% Glycerol. 1 mM DTT, 20 mM β-glycerolphosphate, 0.2 mM sodium orthovanadate, 20 mM p-Nitrophenyl phosphate (PNPP), 1 mM PMSF, 1 mM Benzamidine supplemented with 1 mini protease inhibitor tablet per 10 ml (ROCHE)]. Lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C. 0.5 mg protein lysate was incubated for 3 h at 4° with 7.5 mg of IgG-purified anti-IKKε antibody, followed by 1 h incubation at 4° with 20 ml of protein G slurry (Amersham). Samples were rotated end-over-end during all incubations. Protein G beads were pre-blocked and equilibrated with lysis buffer containing 0.2 mg/ml BSA. After the immunoprecipitation, the beads were collected by centrifugation for 3 min at 6,000 rpm and washed twice with lysis buffer. Kinase assays with biotinylated IRF-3 peptide aa 380–406 (GGASSLENTVDLHISNSHPLSLTSDQ) as substrate were performed out in a total volume of 10 ml in kinase buffer containing 100 mM ATP, 1.0 mg of substrate and 0.33 μCi of ^{32}P-γATP. The reaction was carried out for 30 min at 37° and was terminated by addition of 20 ml of 7.5 M guanidine hydrochloride. The phosphorylated substrate was spotted on SAM2, a biotin capture membrane (Promega, Madison, WI). Filters were washed several times with 2 M NaCl solution and phosphorylated substrate was visualized and analyzed by PhosphoImager after overnight exposure.

Note added in proof

While the manuscript was in revision, Chiang et al. published that LPS-induced IRF-3 activation also occurs through NADPH-mediated ROS formation (Journal of Immunology 2006, 176:5720–24).

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