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Essential Role for the dsRNA-Dependent Protein Kinase PKR in Innate Immunity to Viral Infection

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Summary

The double-stranded (ds) RNA-dependent protein kinase PKR is considered to play an important role in interferon's (IFN's) response to viral infection. Here, we demonstrate that mice lacking PKR are predisposed to lethal intranasal infection by the usually innocuous vesicular stomatitis virus, and also display increased susceptibility to influenza virus infection. Our data indicate that in normal cells, PKR primarily prevents virus replication by inhibiting the translation of viral mRNAs through phosphorylation of eIF2α, while concomitantly assisting in the production of autocrine IFN and the establishment of an antiviral state. These results show that PKR is an essential component of innate immunity that acts early in host defense prior to the onset of IFN counteraction and the acquired immune response.

Introduction

The interferons (IFNs) are a family of related pleiotropic cytokines with potent antiviral, immunomodulatory, and antiproliferative activities that exert their multiple effects through the induction of >30 responsive genes (Stark et al., 1998). The IFNs are classified into two major categories referred to as type I (α, β) and type II (γ). Type I IFNs are induced by most cell types in response to viruses or double-stranded (ds) RNA, while type II IFN is mainly expressed by activated T lymphocytes and natural killer cells in response to various growth factors and cytokines (Sen and Ransohoff, 1998). Following expression, the IFN proteins are secreted, bind to species-specific cell surface receptors, and trigger the transcription of genes through activation of Jak-STAT signaling pathways (Darnell et al., 1994).

The importance of IFN in antiviral host defense has been demonstrated by a number of methods, most notably through the use of genetically engineered mice that lack key components of the IFN-signaling pathway. For example, mice lacking functional type I IFN receptors or the key signaling molecule STAT1 have been shown to be extremely sensitive to infection with a number of viruses, including vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), and various strains of influenza virus (Muller et al., 1994; Durbin et al., 1996; Meraz et al., 1996; Garcia-Sastre et al., 1998). However, while these experiments clearly demonstrate the importance of the IFN system in combating viral infection, the IFN-responsive genes accountable for these actions remain to be fully characterized. Although IFN-inducible proteins exhibiting antiviral activity include the 2–5(A) synthetase/RNase L enzymes and the Mx family, these proteins only appear to influence the regulation of certain classes of virus, emphasizing that different antiviral mechanisms may be mediated by a diverse array of IFN-induced proteins (Stark et al., 1998).

The double-stranded (ds) RNA-dependent serine/threonine protein kinase PKR is another IFN-inducible gene that has been proposed to play a role in antiviral host defense (Meurs et al., 1990). PKR is constitutively expressed in the absence of IFN induction and exists as a latent 68 kDa or 65 kDa molecule in human and murine cells, respectively. Following interaction with dsRNA, PKR autophosphorylates and, in turn, phosphorylates substrate targets, the best characterized being the α subunit of eukaryotic protein synthesis initiation factor 2 (eIF2α) (Panniers and Henshaw, 1983). Phosphorylation of eIF2α on serine 51 causes a dramatic inhibition of protein synthesis in the cell by sequestering the guanine nucleotide exchange factor eIF2B, a rate-limiting component of the translation machinery. Sequestered eIF2B prevents the exchange of GDP for GTP on eIF2α and inhibits the initiation of protein synthesis (Hershey, 1991). In addition to this role, PKR has also been reported to function in a variety of signaling pathways, including those involving dsRNA, PDGF, and NF-κB (Mundschau and Faller, 1995; Yang et al., 1995; Der et al., 1997; Kumar et al., 1997; Chu et al., 1999). Recent evidence from our group and others have also demonstrated that activation of PKR can induce the expression of Fas and trigger apoptosis through the FADD/caspase-8 death signaling pathway (Balachandran et al., 1998; Donze et al., 1999). In contrast, overexpression of a catalytically inactive PKR variant renders immortalized cells resistant to a number of apoptotic stimuli and induces their malignant transformation (Koromilas et al., 1992; Meurs et al., 1990; Balachandran et al., 1998). Unsurprisingly, to neutralize the deleterious effects that activation of PKR would have upon viral replication, numerous viruses, including vaccinia virus, adenovirus, hepatitis C, and human immunodeficiency virus type I, appear to have developed strategies to suppress this kinase (Katze, 1993).

Despite a plethora of evidence implicating a role for PKR in host defense, mice with targeted disruptions in the PKR gene have not yet been reported to exhibit any significant defect in immunity to viral infection. Although the antiviral effects of IFN-γ against EMCV infection were reportedly impaired, host responses to a number of viruses including vaccinia virus were described as

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normal (Yang et al., 1995; Abraham et al., 1999). Indeed, mice doubly defective in both PKR and RNase L showed only partial defects in response to EMCV infection (Zhou et al., 1999). Although these data highlight possible redundancies in the IFN antiviral system and accentuate the existence of alternate key antiviral genes, it is plausible that the type of virus, route of infection, and even genetic strain of the host complicates the evaluation of genes considered important in host defense.

Taking this into consideration, we have extended the evaluation of PKR's role in innate immunity to viral infection. Our data indicate that PKR-deficient mice but not wild-type mice are extremely susceptible to lethal VSV and influenza virus (WSN) infection. We further show that PKR inhibits VSV replication at the level of viral translation by phosphorylating eIF2α, while assisting with autocrine IFN production. Although IFN treatment of fibroblasts lacking PKR could prevent VSV replication in vitro, these cytokines could not protect $PKR^{-/-}$ mice from fatal intranasal infection, further suggesting an inoperative IFN system in certain tissues of the respiratory tract. PKR's ability to encumber viral replication is thus nonredundant and likely allows time for the acquired arm of host immunity to respond to and assist in the eradication of the infectious agent.

Results

PKR-Deficient Fibroblasts Contain Low Levels of Phosphorylated eIF2α

To further characterize cells deficient in PKR, primary embryonic fibroblasts (EFs) obtained from mice containing a deletion in the catalytic domain of PKR (Abraham et al., 1999) were treated with or without dsRNA [poly (I:C)] in the presence or absence of mIFN (α/β) and were analyzed for PKR protein expression and kinase activity. As shown in Figure 1, an increase in PKR expression was observed in wild-type EFs treated with IFN (Figure 1A, compare lane 2 to lane 1). In vivo [^{32}P]orthophosphate labeling experiments also showed an increase in PKR phosphorylation levels following dsRNA treatment (Figure 1B, compare lane 3 to lane 2). Importantly, we were unable to detect PKR protein expression or kinase activity in EFs derived from $PKR^{-/-}$ mice (Figures 1A and 1B, lanes 4–6).

We next examined the phosphorylation status of eIF2α in $PKR^{+/+}$ and $PKR^{-/-}$ EFs using antibodies capable of detecting either total or phosphorylated eIF2α protein. While the levels of total eIF2α were essentially equivalent in both types of fibroblasts, there was a significant reduction but not complete ablation of phosphorylated eIF2α in $PKR^{-/-}$ EFs (Figure 1C, compare lane 3 to lane 1). DsRNA treatment did not appear to affect the levels of total eIF2α in EFs from $PKR^{+/+}$ or $PKR^{-/-}$ mice but did cause an increase (almost 2-fold) in the levels of phosphorylated eIF2α only in fibroblasts from $PKR^{+/+}$ mice (Figure 1C, compare lane 1 to lane 2). These results demonstrate that PKR is a key regulator of eIF2α in these cells.

$PKR^{-/-}$ Fibroblasts Are Susceptible to VSV Replication and Caspase 9–Activated Apoptosis

To further evaluate the antiviral role of PKR, primary embryonic fibroblasts obtained from $PKR^{+/+}$ and $PKR^{-/-}$ mice were treated with or without mIFN-α/β or mIFN-γ and subsequently infected with a selection of RNA viruses including VSV. We observed that VSV infection at a multiplicity of infection (moi) of 10 induced cytolysis of $PKR^{-/-}$ EFs with the morphological characteristics of apoptosis by 18 hr postinfection (Figure 2A, panel e). In contrast, $PKR^{+/+}$ EFs remained mostly viable for at least 36 hr postinfection (Figure 2A, panel b). We also found

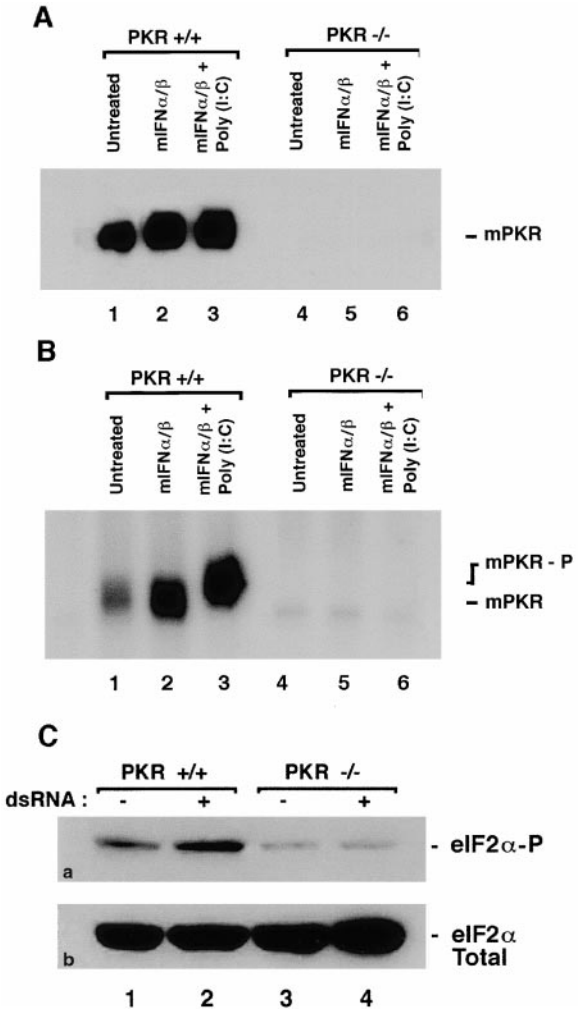


Figure 1. Ablation of PKR in EFs from Mice with a Targeted Disruption of the Catalytic Domain of PKR

(A) EFs from $PKR^{+/+}$ (lanes 1–3) and $PKR^{-/-}$ (lanes 4–6) mice were left untreated (lanes 1 and 4), treated with murine interferon (mIFN-α/β) alone for 18 hr (lanes 2 and 5), or pretreated with mIFN for 18 hr and subsequently transfected with poly (I:C) (lanes 3 and 6). Lysates prepared from these cells were analyzed by Western blotting for the presence of murine PKR (mPKR) using an mPKR-specific antibody.

(B) EFs from $PKR^{+/+}$ and $PKR^{-/-}$ mice treated as above were labeled during the last 4 hr of their treatment with [^{32}P]orthophosphate. mPKR was precipitated from lysates prepared from these cells using poly (I:C)–agarose beads and visualized by autoradiography following SDS-PAGE.

(C) $PKR^{+/+}$ (lanes 1 and 2) and $PKR^{-/-}$ (lanes 3 and 4) EFs were transfected with poly (I:C) (lanes 2 and 4) for 4 hr, or mock-transfected (lanes 1 and 3), lysed, and analyzed by Western blotting for phosphorylated eIF2α (a) or total eIF2α (b) using antibodies specific for eIF2α phosphoserine 51, or total eIF2α, respectively. Identities of the individual proteins are indicated on the right.

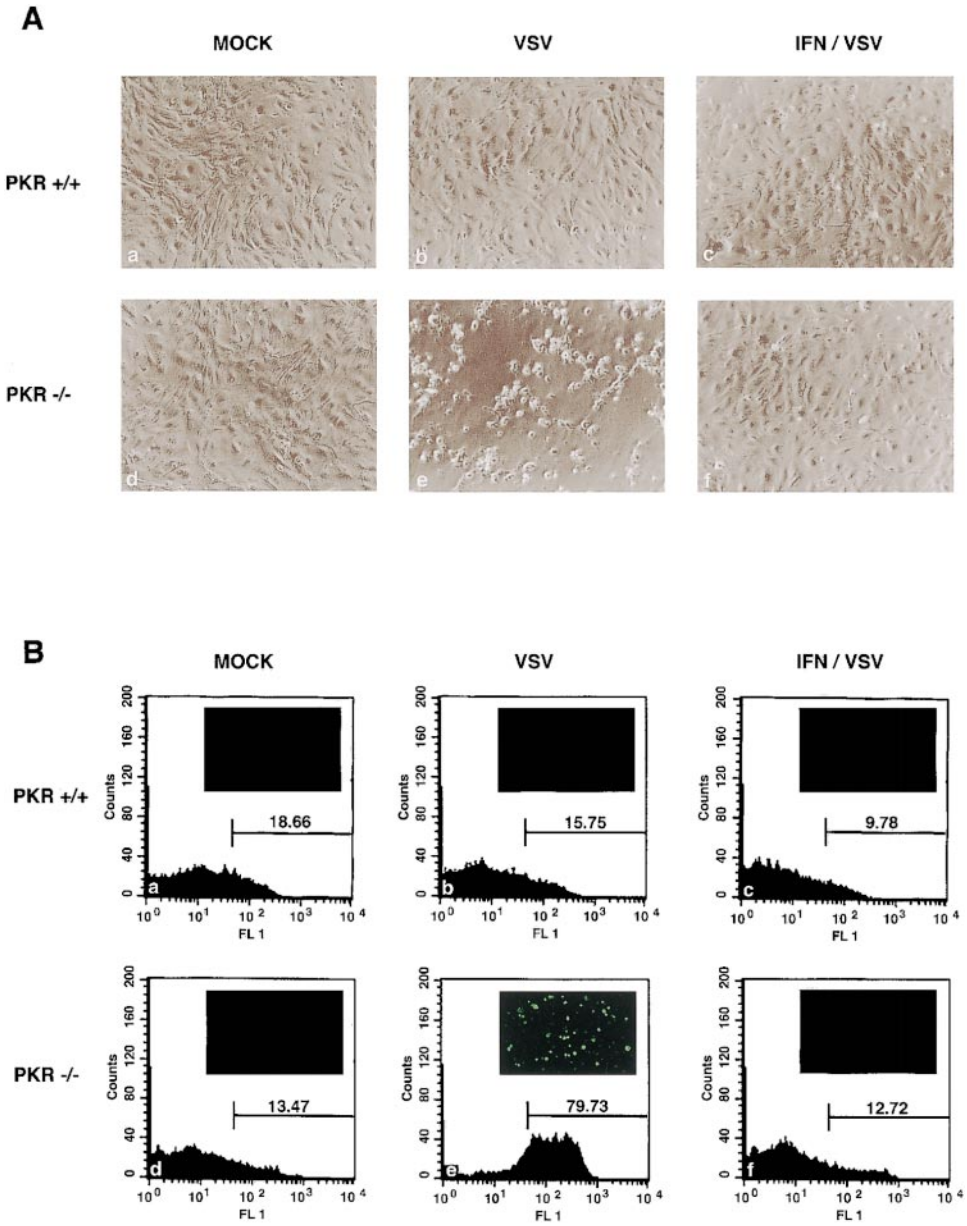


Figure 2. VSV Induces Apoptosis in PKR$^{-/-}$ Primary EFs

PKR$^{+/+}$ (a–c) and PKR$^{-/-}$ (d–f) EFs were treated with (c and f) or without (a, b, d, and e) mIFN-α/β for 18 hr and mock-infected (a and d) or infected with VSV (Indiana strain) at an moi of 10 (b, c, e, and f). Twenty hours postinfection, cells were (A) photographed at 200$\times$ magnification or (B) analyzed for apoptosis using FITC-conjugated annexin V by flow cytometry and TUNEL (inset). Percent cells staining positive for annexin V within the defined region are shown in each panel above the region bar.

that pretreatment with either mIFN-α/β or -γ completely protected the PKR$^{-/-}$ EFs against VSV-induced cell death, indicating that an IFN-induced component capable of inhibiting VSV replication can compensate for the loss of PKR in these cells (Figures 2A, panel f, and 3A).

To establish whether PKR$^{-/-}$ EFs were indeed undergoing apoptosis following VSV infection, PKR$^{+/+}$ and PKR$^{-/-}$ cells infected as above were analyzed for annexin V binding, an early indicator of apoptosis, as well as by TUNEL. As shown in Figure 2B, VSV-infected PKR$^{-/-}$ EFs displayed a marked increase in annexin V binding (79.73% infected versus 13.47% uninfected) and TUNEL staining, while VSV-infected PKR$^{+/+}$ EFs showed no increase in annexin V staining or evidence of DNA fragmentation over mock-infected samples (15.75% infected versus 18.66% uninfected). Interestingly, IFN-treated, VSV-infected PKR$^{-/-}$ EFs were protected against virus-induced apoptosis (Figure 2B). Similar results were obtained when VSV infections were performed at an moi of 1 (data not shown).

We also examined the relative sensitivities of PKR$^{+/+}$ and PKR$^{-/-}$ EFs to dsRNA-mediated apoptosis. We found PKR$^{-/-}$ EFs to be remarkably resistant to dsRNA-triggered cell death ($\sim$10% were dead 24 hr posttreatment), while in contrast, $\sim$80% of the wild-type EFs were dead within 24 hr of dsRNA transfection (Figure 3B).

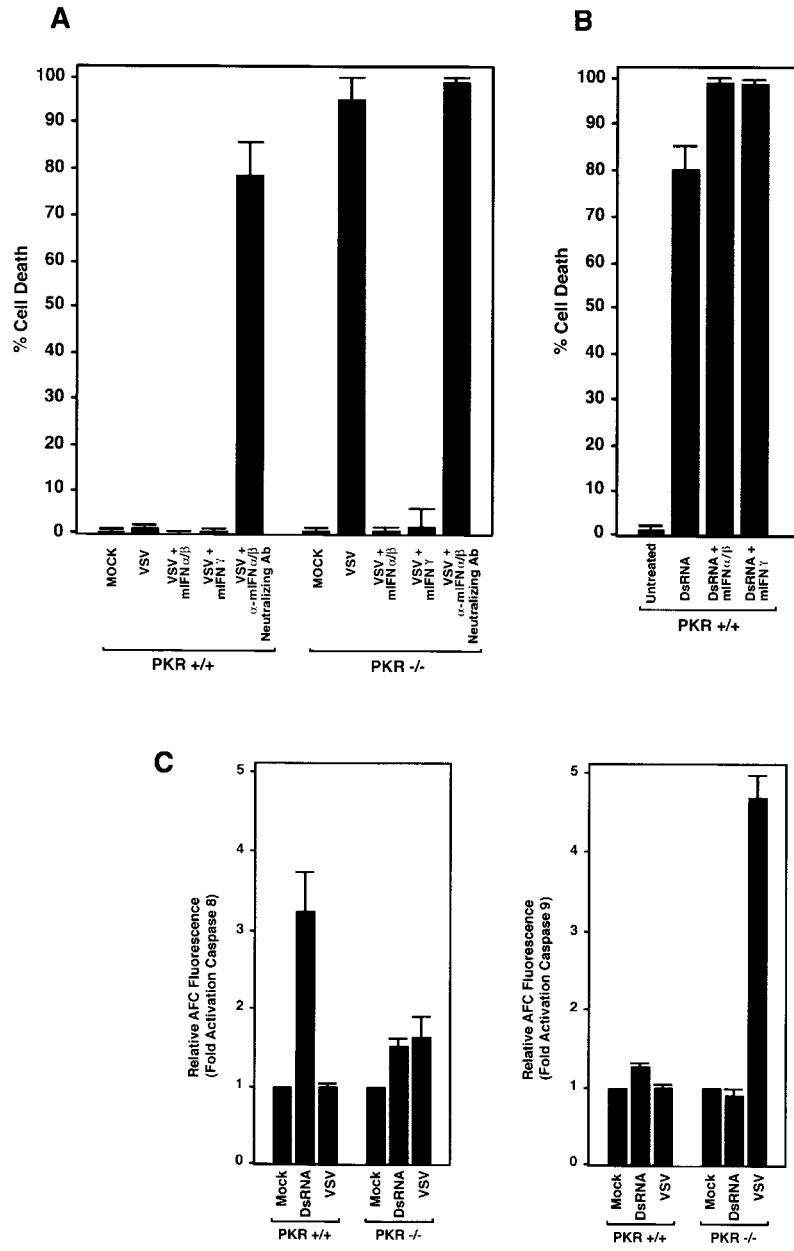


Figure 3. Differential Effects of PKR and IFN on VSV- and dsRNA-Induced Apoptosis in Primary EFs

(A) PKR$^{+/+}$ and PKR$^{-/-}$ EFs were infected with VSV in the presence or absence of either anti-mIFN-α/β neutralizing antibodies or 18 hr mIFN-α/β or mIFN-γ pretreatment. Cell viability was determined 24 hr postinfection by trypan blue exclusion.

(B) PKR$^{+/+}$ and PKR$^{-/-}$ EFs were transfected with poly (I:C) in the presence or absence of 18 hr mIFN-α/β or mIFN-γ pretreatment. Twenty-four hours posttransfection, cell viability was assayed by trypan blue exclusion. Data shown represent the mean ± SD of triplicate samples of one of two experiments with similar results.

(C) PKR$^{+/+}$ and PKR$^{-/-}$ EFs were transfected with poly (I:C) or infected with VSV (moi = 10) and analyzed for caspase 8 or caspase 9 activity 24 hr posttreatment, as described in Experimental Procedures. Data shown represent the average of triplicate samples of one of two independent experiments with similar results.

Since IFN has been reported to sensitize cells to dsRNA-induced cytotoxicity (Stewart et al., 1972), we examined whether priming with either mIFN-α/β or mIFN-γ could render PKR$^{-/-}$ EFs susceptible to dsRNA-induced apoptosis. Accordingly, wild-type and PKR$^{-/-}$ EFs were pretreated with either IFN-α/β or IFN-γ for 18 hr prior to transfection with dsRNA. However, while virtually all the wild-type EFs underwent rapid dsRNA-induced apoptosis following IFN treatment, PKR$^{-/-}$ EFs remained mostly viable, indicating the essential role of PKR in regulating IFN-mediated, dsRNA-induced apoptosis (Figure 3B).

To evaluate the mechanisms of apoptosis further, we examined caspase 8 and 9 activity using fluorogenic

Table 1. Viral Titers from $PKR^{+/+}$ and $PKR^{-/-}$ EFs in the Presence of mIFN-α/β Pretreatment or in the Presence of Neutralizing Anti-mIFN-α/β Antibodies

Genotype	moi	Untreated (pfu/ml)	mIFN-α/β (pfu/ml)	Anti-mIFN-α/β (pfu/ml)
$PKR^{+/+}$	1	1.4×10^3	3.5×10^2	2.5×10^6
$PKR^{+/+}$	10	4.8×10^3	4.7×10^2	2.4×10^7
$PKR^{-/-}$	1	6.4×10^7	3.5×10^4	8.8×10^8
$PKR^{-/-}$	10	7.3×10^7	8.3×10^4	1.7×10^9

Numbers represent mean of duplicate samples from two independent experiments. Individual titers did not vary by more than one log.

tetrapeptide substrates specific for each caspase in a fluorometric assay. As we have previously shown in wild-type cells, dsRNA treatment results in the activation of caspase 8 (Figure 3C; Balachandran et al., 2000). In contrast, $PKR^{-/-}$ EFs did not exhibit any significant increase in caspase 8 activity following dsRNA treatment. Our data also show that VSV infection predominantly causes the activation of caspase 9 rather than of caspase 8, and primarily in the PKR-deficient fibroblasts. Thus, VSV induces apoptosis through a mechanism that appears different from that triggered by dsRNA, and that proceeds, at least in part, through the activation of caspase 9.

We next determined whether the induction of apoptosis by VSV in $PKR^{-/-}$ cells correlated with an increase in viral replication. Virus progeny yield from $PKR^{+/+}$ and $PKR^{-/-}$ EFs previously treated with or without mIFN-α/β and infected with VSV at an moi of 1 or 10 were measured 36 hr postinfection. While $PKR^{+/+}$ cells were found to be essentially nonpermissive to VSV (Table 1, 1.4×10^3 pfu/ml virus yields at an moi of 1), $PKR^{-/-}$ cells proved quite capable of sustaining VSV replication, and yields corresponding to 6 to 7×10^7 pfu/ml were routinely at an moi of 1 (Table 1). Although IFN pretreatment was able to significantly decrease virus yields from $PKR^{-/-}$ EFs, these cytokines were not able to completely restore these cells to the nonpermissive state seen in $PKR^{+/+}$ EFs (Table 1, 3.5×10^4 pfu/ml yield from IFN-treated $PKR^{-/-}$ EFs versus $\sim 3.5 \times 10^2$ pfu/ml from IFN-treated $PKR^{+/+}$ EFs).

IFN Produced during VSV Infection Is Required for the Complete Protection of $PKR^{+/+}$ Fibroblasts

It is known that cells lacking a functioning IFN system, such as IFN-α/β receptor- and STAT1-deficient EFs, are also susceptible to VSV infection, even though PKR is presumably present in these cells (Muller et al., 1994; Durbin et al., 1996; Meraz et al., 1996). We therefore speculated that PKR may function at an early step in cellular antiviral defense, primarily to delay the translation of viral mRNAs and consequent virion production long enough for IFN to be produced. IFN secreted from the infected cell would then have time to act in an autocrine fashion, inducing other genes required for the establishment of a complete antiviral state.

To address the role of autocrine IFN produced during the course of VSV infection, we infected $PKR^{+/+}$ and $PKR^{-/-}$ EFs with VSV (moi = 10) in the presence of neutralizing anti-IFN-α/β antibodies, which inhibit IFN action by preventing ligation to their receptors. Strikingly, we found that neutralizing anti-IFN-α/β antibody treatment rendered normal fibroblasts containing PKR susceptible to VSV replication and cell death (Table 1; Figure 3A). In fact, viral yields from $PKR^{+/+}$ cells treated with neutralizing anti-IFN-α/β antibodies had now increased into the range of those observed from $PKR^{-/-}$ cells in the absence of antibody treatment (Table 1). Interestingly, $PKR^{-/-}$ cells cultured in the presence of neutralizing anti-IFN-α/β antibodies after VSV infection yielded 10-fold more virus than VSV-infected $PKR^{-/-}$ cells (Table 1). This suggests that VSV can induce some IFN in $PKR^{-/-}$ cells able to modestly affect viral replication even in the absence of PKR. Nevertheless, these data clearly demonstrate a nonredundant role for PKR in the IFN response to VSV infection and demonstrate the synergistic nature of IFN and PKR in maintaining an antiviral state in the cell.

PKR Inhibits VSV Protein Synthesis

In an attempt to analyze the mechanism by which PKR inhibits VSV replication, we infected $PKR^{+/+}$ and $PKR^{-/-}$ EFs with VSV in the presence of [^{32}P]orthophosphate and examined the in vivo phosphorylation status of PKR. As seen in Figure 4A, PKR clearly becomes activated by VSV following infection of the cell (Figure 4A, compare lanes 2 and 4 to lanes 1 and 3). However, as expected, no phosphorylated 65 kDa protein corresponding to PKR can be seen in precipitates from $PKR^{-/-}$ EFs following VSV infection and/or IFN treatment (Figure 4A, lanes 5–8).

VSV-infected $PKR^{+/+}$ and $PKR^{-/-}$ EFs were concomitantly analyzed for PKR protein expression and levels of phosphorylated and unphosphorylated eIF2α. As shown in figure 4B, IFN treatment caused a slight increase in PKR protein levels in $PKR^{+/+}$ EFs (panel a, compare lanes 3 and 4 to lanes 1 and 2), although as previously demonstrated, no PKR was detected in lysates prepared from $PKR^{-/-}$ EFs. Importantly, we also observed an increase in the level of phosphorylated eIF2α in VSV-infected $PKR^{+/+}$ EFs (Figure 4B, panel b, compare lanes 2 and 4 to lanes 1 and 3). This increase was not seen in similarly treated $PKR^{-/-}$ EFs (Figure 4B, panel b, compare lanes 6 and 8 to lanes 5 and 7). In fact, a slight decrease in the amount of phosphorylated eIF2α was observed in the VSV-infected $PKR^{-/-}$ EFs compared to the levels in mock-infected cells of the same genotype. Equivalent levels of total eIF2α and tubulin confirm that approximately equal amounts of total protein are present in each lane (Figure 4B, panels c and d). These data clearly demonstrate that PKR is activated and phosphorylates eIF2α during VSV infection. It is further interesting to note that although IFN pretreatment was able to prevent VSV replication in PKR-deficient fibroblasts, the absence of any increase in eIF2α phosphorylation levels in IFN-treated, VSV-infected $PKR^{-/-}$ cells indicates that IFN rescues PKR-deficient cells from cytopathic, productive VSV infection through mechanisms independent of eIF2α (Figure 4B, panel b, compare lane 8 to lane 7).

To determine whether the observed activation of PKR and consequent phosphorylation of eIF2α following VSV infection of $PKR^{+/+}$ EFs would have an inhibitory effect

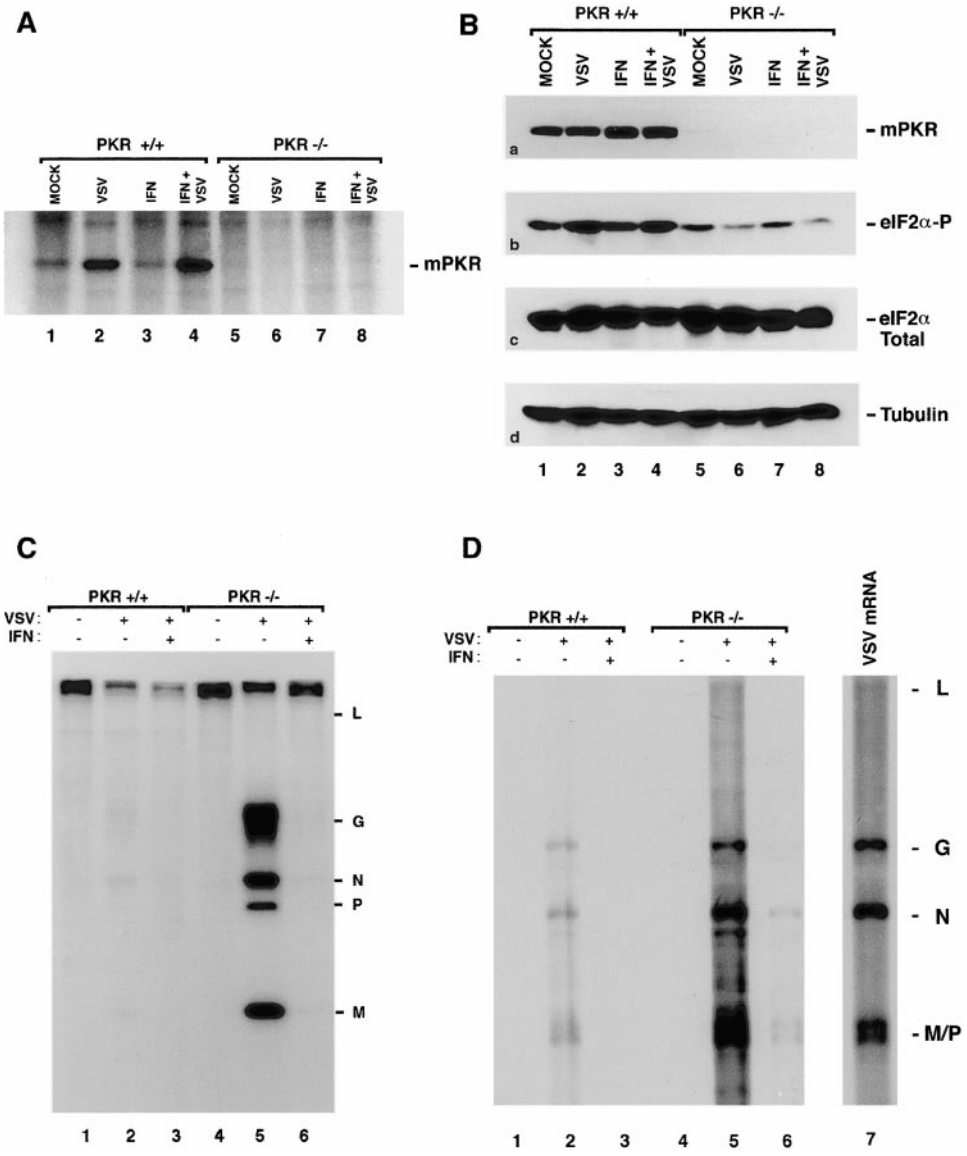


Figure 4. Activation of PKR and Phosphorylation of eIF2α in PKR$^{+/+}$ EFs Inhibit VSV mRNA Translation

(A) PKR$^{+/+}$ (lanes 1–4) and PKR$^{-/-}$ (lanes 5–8) EFs pretreated with mIFN-α/β (lanes 3, 4, 7, and 8) or left untreated (lanes 1, 2, 5, and 6) were infected with VSV at an moi of 500 (lanes 2, 4, 6, and 8), or mock-infected (lanes 1, 3, 5, and 7) and labeled with [^{32}P]orthophosphate for 4 hr in phosphate-free DMEM containing 2% dialyzed FBS. Lysates were prepared from these cells and mPKR was precipitated with poly (I:C)–agarose, resolved by SDS-PAGE, and visualized by autoradiography.

(B) PKR$^{+/+}$ and PKR$^{-/-}$ EFs were treated as above, with the exception that they were incubated in complete DMEM in the absence of [^{32}P]orthophosphate for 4 hr. Lysates obtained from these cells were subjected to Western blot analysis for mPKR (a), phosphorylated eIF2α (b), and total eIF2α (c). Tubulin levels (d) show equivalent amounts of protein in each lane. The identities of the individual proteins are indicated on the right.

(C) PKR$^{+/+}$ (lanes 1–3) and PKR$^{-/-}$ (lanes 4–6) either pretreated with mIFN-α/β (lanes 3 and 6) or untreated (lanes 1, 2, 4, and 5) were infected with VSV at an moi of 10 (lanes 2, 3, 5, and 6) or were mock-infected (lanes 1 and 4). These cells were then labeled with 100 μCi [^{35}S]methionine/cysteine per ml for 6 hr. Lysates prepared from these cells were immunoprecipitated with anti-VSV antibodies, and VSV proteins were detected by autoradiography following SDS-PAGE. Identities of individual VSV proteins are shown on the right.

(D) PKR$^{+/+}$ (lanes 1–3) and PKR$^{-/-}$ (lanes 4–6) EFs were pretreated (lanes 3 and 6) with mIFN-α/β at 1000 U/ml for 18 hr or were left untreated (lanes 1, 2, 4, and 5) and subsequently mock-infected (lanes 1 and 4) or infected with VSV (lanes 2, 3, 5, and 6) at an moi of 500. Following infection, cells were labeled with 50 μCi per ml of [^{3}H]uridine for 6 hr in the presence of 10 μg/ml actinomycin D. RNA was extracted from lysates prepared from these cells and separated by agarose-urea gel electrophoresis. Viral mRNAs were visualized by fluorography. The control VSV mRNA sample (lane 7) represents VSV mRNAs obtained from infecting BHK-21 cells with VSV in the presence of [^{3}H]uridine and actinomycin D and shows the relative sizes of the VSV transcripts. The identities of the individual VSV mRNA species are indicated on the right.

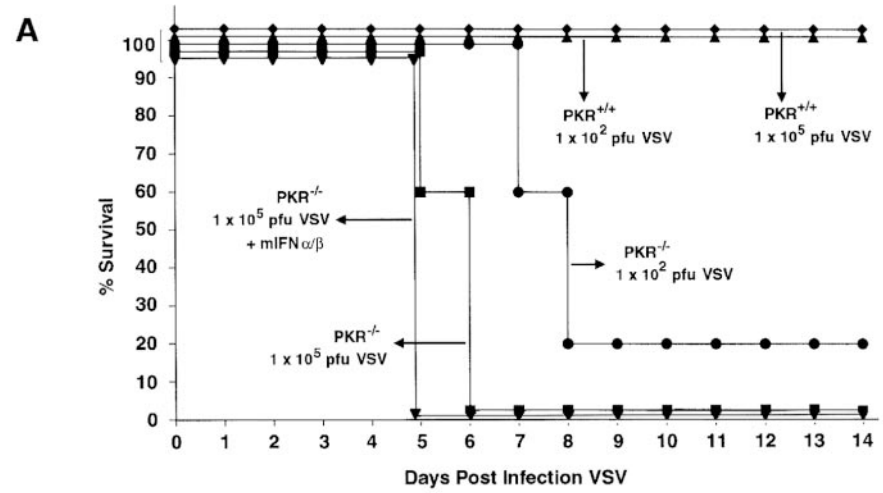
on viral protein translation, we followed the synthesis of VSV proteins by metabolic labeling after infection. Figure 4C shows that VSV protein synthesis is dramatically increased in $PKR^{-/-}$ EFs compared to $PKR^{+/+}$ EFs (compare lanes 2 and 5). However, IFN pretreatment of $PKR^{-/-}$ cells was able to reduce VSV protein synthesis rates to the virtually undetectable levels seen in untreated or IFN-treated wild-type fibroblasts (Figure 4C, compare lane 6 to lanes 2 and 3). To examine whether the inhibition of protein synthesis was due to a reduction in viral mRNA synthesis, $PKR^{+/+}$ and $PKR^{-/-}$ cells were infected with VSV in the presence of [^{3}H]uridine and actinomycin D to suppress cellular RNA synthesis. This analysis showed that VSV mRNA synthesis was significantly reduced but not ablated in wild-type cells compared to PKR-deficient cells (Figure 4D, compare lanes 2 and 5). We reason that the reduction in VSV mRNA synthesis in $PKR^{+/+}$ cells is at least in part due to the unavailability of viral proteins (as a result of the translational block imposed by PKR) for viral genome replication, which is required for amplification of mRNA transcription in VSV-infected cells. Initial transcription from input genomic templates by the RNA-dependent RNA polymerase carried within the virion into the cell (Wagner and Rose, 1996) is likely responsible for the small amount of viral mRNA transcription seen in the $PKR^{+/+}$ cells. Indeed, analysis of VSV mRNA transcription in $PKR^{+/+}$ and $PKR^{-/-}$ cells in the presence of the protein synthesis inhibitor cycloheximide showed that transcription from input genomic templates in $PKR^{+/+}$ and $PKR^{-/-}$ cells were comparable, indicating that viral transcription per se is not affected in cells containing PKR (data not shown). It is noteworthy that IFN-treated cells were also defective in viral mRNA production (Figure 4D). Since there was no increase in the levels of phosphorylated eIF2α in the IFN-treated $PKR^{-/-}$ cells following VSV infection, the mechanism by which IFN suppresses viral protein and/or mRNA synthesis in the absence PKR is likely independent of eIF2α.

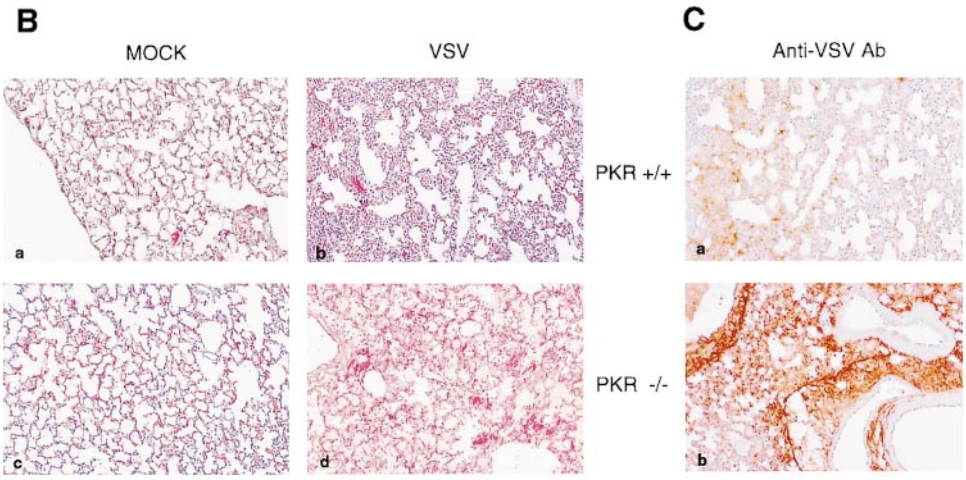
Susceptibility of $PKR^{-/-}$ Mice to Intranasal Infection by VSV and Influenza Virus

To determine the importance of PKR antiviral activity against VSV infection in vivo, PKR-deficient or genotype-controlled normal mice were inoculated by different routes with varying amounts of VSV. Neither intravenous (i.v.) nor intraperitoneal (i.p.) injections of up to 1×10^6 pfu VSV caused any morbidity in either $PKR^{+/+}$ or $PKR^{-/-}$ mice (data not shown). In contrast, intranasal (i.n.) inoculation of VSV was found to be extremely lethal to $PKR^{-/-}$ mice but not to wild-type mice (BALB/c, 129terSv, 129terSv $\times$ BALB/c). In fact, as few as 100 pfu VSV killed most of the PKR null mice within 8 days, and 1×10^5 pfu VSV proved lethal to all PKR-deficient animals by day 6 (Figure 5A). These mice displayed marked respiratory distress and succumbed to paralytic disease. $PKR^{+/+}$ mice, however, suffered no mortality and exhibited no overt signs of sickness even after i.n. infection with 1×10^6 pfu/mouse VSV. Examination of viral loads in the tissues of the infected animals indicated that VSV replicated to high titers primarily in the lungs and brain of the $PKR^{-/-}$ mice (Figure 5D).

Histological examination of hematoxylin/eosin-stained sections of lung tissue from VSV-infected $PKR^{-/-}$ mice demonstrated severe edema and congestion of the alveoli without any obvious inflammatory cells present in either the exudate or the alveolar wall (Figure 5B, panel d). Interestingly, only lungs from $PKR^{+/+}$ mice infected i.n. with VSV showed signs of inflammation, with interstitial pneumonitis, thickening of alveolar walls, and proliferation of pneumocytes (Figure 5B, panel b). This was accompanied by infiltration of histiocytes, lymphocytes, and neutrophils. In the absence of viral infection, however, no immune system abnormalities were overtly evident in mice lacking PKR (splenocytes: 26.8% $CD4^+$, 12.7% $CD8^+$, 50.3% $B220^+$ in $PKR^{-/-}$ mice versus 26.7% $CD4^+$, 13.5% $CD8^+$, 55.7% $B220^+$ in $PKR^{+/+}$ mice; thymocytes: 9.0% $CD4^+$, 2.5% $CD8^+$, 86.4% CD4/CD8 double-positive in $PKR^{-/-}$ mice versus 9.5% $CD4^+$, 2.6% $CD8^+$, 85.8% CD4/CD8 double-positive in $PKR^{+/+}$ mice; data not shown). Staining of lung sections from VSV-infected $PKR^{+/+}$ and $PKR^{-/-}$ mice for viral proteins using anti-VSV antibodies clearly showed significant VSV replication in the lungs of $PKR^{-/-}$ mice (Figure 5C). Very little evidence of VSV antigen was seen in infected lungs retrieved from $PKR^{+/+}$ mice. Curiously, no significant apoptosis, as determined by TUNEL and caspase activation assays, could be detected in the lungs of PKR-deficient mice infected with VSV for reasons that presently remain unknown (data not shown). To determine if prophylactic administration of IFN could protect the $PKR^{-/-}$ mice against lethal VSV infection, 25,000 U of IFN was administered i.p. as well as i.n. 18 hr prior to VSV infection and 12 and 48 hr postinfection. This treatment was not able to prevent lethal VSV infection, although it did cause a significant reduction in viral yield from both the lungs and the brains of PKR-deficient mice (Figure 5D). Since IFN was able to protect $PKR^{-/-}$ EFs against productive VSV infection in vitro, it is possible that tissues comprising the respiratory tract may be less responsive to IFN (Ronni et al., 1997).

Given these findings, it was plausible that $PKR^{-/-}$ mice would be compromised in their immunity to i.n. infection by other viruses. Therefore, we next examined the effects of influenza virus infection in PKR-deficient and wild-type mice. The mouse-adapted influenza A/WSN/33 strain of the virus was used for this purpose. We found that, similar to VSV, WSN did not cause any mortality when inoculated i.v. (data not shown). However, $PKR^{-/-}$ mice were significantly more susceptible to i.n. WSN infection, and all infected mice died at doses of 1×10^5 pfu/mouse within 6 days (Figure 6A). In fact, three out of five $PKR^{-/-}$ mice succumbed to i.n. infection with as low as 1×10^4 pfu/mouse, while none of the wild-type mice succumbed to this dose of virus (Figure 6A). Viral yields obtained from the lungs of infected mice showed $\sim$7-fold greater levels of WSN in $PKR^{-/-}$ versus $PKR^{+/+}$ animals (Figure 6D). Interestingly, we also noticed significant apoptosis in the lungs of WSN-infected animals, particularly in PKR-deficient mice. Aside from prominent TUNEL staining (Figure 6B), the lungs from WSN-infected $PKR^{-/-}$ mice had higher levels of both active caspase 8 and 9 compared to wild-type mice (Figure 6C), implying that the virus (either inadvertently or deliberately) activates apoptosis in infected cells during the course of its replication. WSN infection of EFs





D

GENOTYPE	IFN	TISSUE	
		Brain	Lung
PKR +/+	-	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
PKR +/+	+	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
PKR -/-	-	5.0×10^5	2.0×10^6
PKR -/-	+	6.5×10^3	7.5×10^4

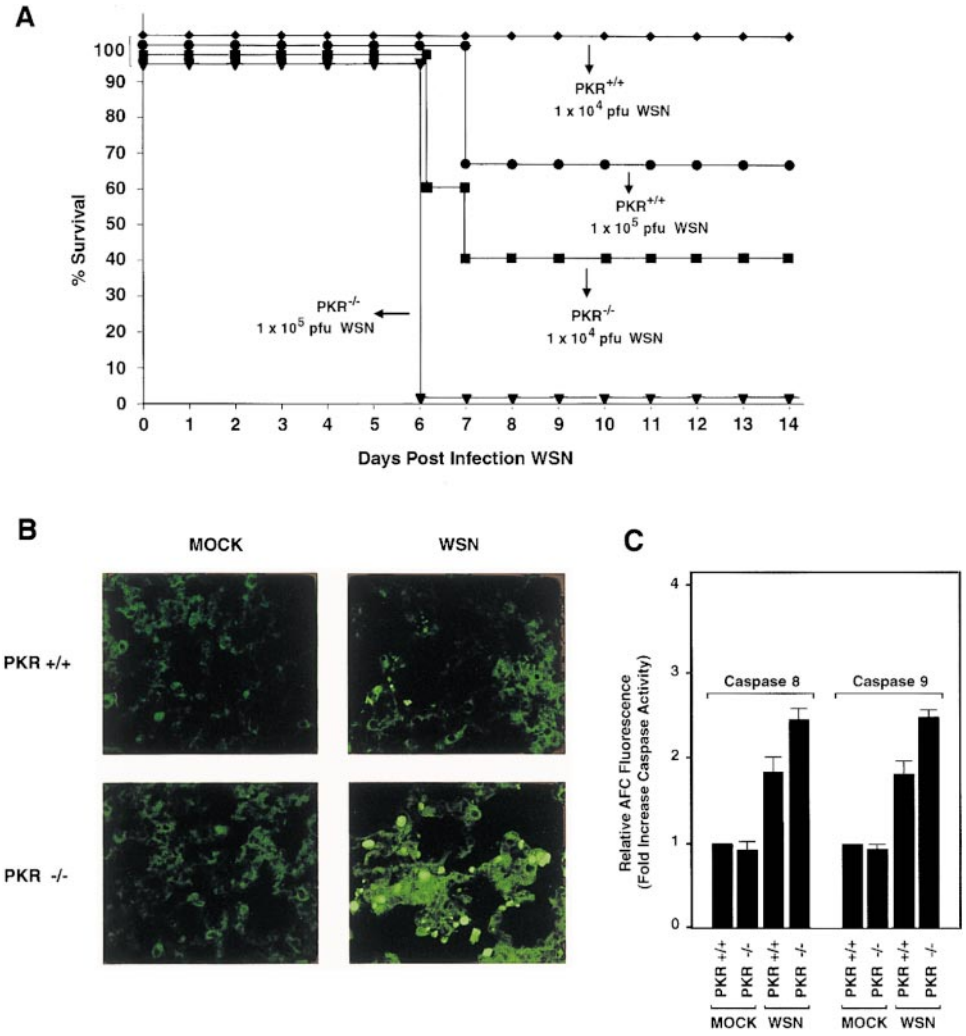
Figure 5. Mice Lacking PKR Are Very Susceptible to VSV Replication and Lethality

(A) $PKR^{+/+}$ and $PKR^{-/-}$ mice were infected i.n. with 1×10^5 pfu VSV or 1×10^2 pfu VSV per mouse with or without prophylactic IFN treatment. Mice were monitored daily for up to 14 days, and animals surviving at the indicated time points are plotted versus time. Five to six mice per genotype were used for each condition.

(B) Paraffin-embedded lung sections taken from mock-infected (a and c) or VSV-infected (b and d) $PKR^{+/+}$ and $PKR^{-/-}$ mice were stained with hematoxylin/eosin and photographed at 100× magnification. Severe edema is evident in VSV-infected $PKR^{-/-}$ lungs (d), whereas marked infiltration is seen in similarly infected $PKR^{+/+}$ cells (c).

(C) $PKR^{+/+}$ (a) and $PKR^{-/-}$ (b) lung sections were stained for VSV antigens using a polyclonal antiserum that recognizes all VSV proteins. Dense staining is present only in the $PKR^{-/-}$ section.

(D) $PKR^{+/+}$ and $PKR^{-/-}$ mice were infected i.n. with 1×10^5 pfu/mouse VSV. Titers from organs were determined 5 days postinfection by standard plaque assay on BHK-21 cells. Numbers represent the mean titers of duplicate samples from two mice per condition. Individual titers did not vary by more than one log.



D

GENOTYPE	TISSUE	
	Brain	Lung
PKR +/+	$< 1.0 \times 10^2$	4.2×10^5
PKR -/-	$< 1.0 \times 10^2$	2.7×10^6

Figure 6. PKR$^{-/-}$ Mice Show Increased Susceptibility to Intranasal Infection by WSN

(A) PKR$^{+/+}$ and PKR$^{-/-}$ mice were infected i.n. with 1×10^5 pfu or 1×10^4 pfu WSN per mouse. Mice were monitored daily for up to 14 days, and animals surviving at the indicated time points are plotted versus time. Five to six mice per genotype were used for each condition.

(B) Lung tissue taken from mock infected or WSN-infected PKR$^{+/+}$ and PKR$^{-/-}$ mice were assayed for DNA fragmentation by TUNEL, or (C) analyzed for caspase 8 and 9 activity, as described in Experimental Procedures. Data shown represent the mean of duplicate samples from two mice per condition.

(D) PKR$^{+/+}$ and PKR$^{-/-}$ mice were infected i.n.with 1×10^5 pfu/mouse WSN. Titers from organs were determined 6 days postinfection by standard plaque assay on MDCK cells in the presence of trypsin. Numbers represent the mean titers of duplicate samples from two mice per condition. Individual titers did not vary by more than one log.

from PKR$^{-/-}$ and wild-type mice did not result in significant differences in cytopathicity at all moi's tested (data not shown), but ~10-fold higher virus yields were also obtained from PKR$^{-/-}$ EFs than from wild-type EFs (data not shown). Collectively, our data demonstrate that PKR is critical for protection against i.n. infection by VSV and WSN.

Discussion

A number of studies have indicated that PKR plays a key role in IFN-mediated host defense against viral infection. However, to date no reports exist to demonstrate in vivo an essential and nonredundant role for PKR in viral immunity. Here we provide unambiguous evidence

showing that cells and mice devoid of PKR lack defensive capabilities against VSV at usually nonlethal doses and show significantly increased sensitivity to influenza virus.

Our data indicate that PKR prevents replication of VSV in the early stages of the viral replicative cycle, probably after initial transcription of the viral mRNAs from the negative sense RNA genome template by the RNA-dependent RNA polymerase carried by the virus. In wild-type cells, PKR was activated following infection with VSV, an effect that coincided with an increase in phosphorylated eIF2α compared to uninfected cells. It is noteworthy that compared to $PKR^{+/+}$ fibroblasts, lower basal levels of phosphorylated eIF2α were observed in $PKR^{-/-}$ fibroblasts, implying that PKR may be a regulator of eIF2α phosphorylation, even in the absence of viral infection. Other eIF2α kinases such as PERK/PEK (Harding et al., 1999; Shi et al., 1998) the murine homolog of *S. cerevisiae*, GCN2 (Berlanga et al., 1999), or HRI (Berlanga et al., 1998) may be responsible for the residual phosphorylated eIF2α observed in $PKR^{-/-}$ cells. However, since eIF2α phosphorylation did not increase in $PKR^{-/-}$ cells compared to wild-type cells following either transfection of dsRNA or infection with VSV, our data strongly indicate that PKR is the predominant eIF2α kinase activated following viral infection. In fact, a perceptible decrease in the levels of phosphorylated (but not total) eIF2α was observed following VSV infection of $PKR^{-/-}$ cells, the reasons for which are unclear. These observations complemented data showing that VSV protein synthesis occurred to an appreciable degree only in cells lacking PKR, in which no increase of eIF2α phosphorylation was seen. $PKR^{+/+}$ cells also displayed about 10-fold lower levels of VSV RNA synthesis compared to $PKR^{-/-}$ cells, probably because synthesis of viral protein (which is inhibited in wild-type cells) is required for the amplification of viral mRNA seen in the $PKR^{-/-}$ cells.

The importance of PKR in preventing VSV infection was underscored by the fact that mice lacking this kinase are extremely susceptible to lethal i.n. VSV infection. VSV was shown to replicate to high titers in $PKR^{-/-}$ primary EFs, as well as in the lungs and brain of PKR-deficient mice. Examination of the lungs of VSV-infected $PKR^{-/-}$ mice revealed severe alveolar congestion and edema. It is therefore likely that in combination with encephalitis and paralytic disease resulting from viral replication in the central nervous system, pulmonary failure may have contributed to the death of these mice.

In EFs derived from $PKR^{-/-}$ mice, VSV infection triggered caspase 9–activated apoptosis. It is not yet clear whether the virus actively triggers apoptosis of the cell to enhance its release and systemic dissemination or whether the induction of apoptosis in the virally infected cell is a host defense mechanism that prevents establishment of a persistent infection (Levine et al., 1993; Albert et al., 1998). Interestingly, in tissue extracts from VSV-infected mice, we were unable to detect significant signs of apoptosis by assays designed to detect either DNA fragmentation or activation of various caspases, indicating that cytolysis as a direct result of viral replication may possibly be responsible for cell death in vivo. Alternatively, it is feasible that an analysis of infected tissue earlier after infection, rather than after 5–7 days as described here, may yield more evidence of apoptosis in the infected animals. Finally, it is further possible that cell-specific factors may also dictate whether a cell will undergo apoptosis or not in response to a particular stimuli. In this regard Sindbis virus vectors carrying pro-apoptotic Bax were found to induce accelerated apoptotic death in several cultured cell lines but triggered significantly less neuronal apoptosis in vivo compared to control virus (Lewis et al., 1999). Nevertheless, that VSV induces cytolysis of nearly all malignant cells analyzed speculatively indicates that PKR function may be affected in these types of cells. It is noteworthy that this information has recently been exploited by our laboratory to demonstrate that VSV can selectively eliminate a variety of tumors in murine models and may thus be useful as an oncolytic virus in the treatment of cancer (S. B. and G. B., unpublished data).

Another difference in response to VSV infection in vitro versus in vivo was observed when IFN pretreatment preceded infection. It was found that priming with IFN-α/β or -γ could effectively compensate for the loss of PKR and prevent VSV replication in PKR-deficient fibroblasts but not in vivo. Inhibition of viral replication by type I and II IFN in EFs occurred without invoking an apoptotic response and was independent of eIF2α phosphorylation. Although it is not yet clear which IFN-responsive genes are responsible for compensating for PKR action, it has been shown that fibroblasts and animals with a defective IFN system (i.e., lacking STAT1 or the IFN-α/β receptor) are extremely sensitive to VSV, as well as to other types of viral disease (Muller et al., 1994; Durbin et al., 1996; Meraz et al., 1996). Since PKR presumably exists in STAT1-deficient cells and in most cell types in a latent form in the absence of IFN stimulation, other IFN-induced genes besides PKR must be essential for preventing VSV replication. However, our data indicate that PKR may provide a crucial first line of defense against certain types of viral infection by delaying the production of progeny virions through the inhibition of viral mRNA translation prior to the induction of IFN. IFN, acting in an autocrine fashion, would then induce other genes necessary to fortify the antiviral state. Indeed, we show in this study that treatment of wild-type cells with neutralizing anti-IFN antibodies following VSV infection rendered these cells permissive to viral replication, even though PKR was present in these cells. In the absence of PKR's translational block, VSV presumably replicates to high levels before the induction of IFN, at which time IFN-induced antiviral host gene products may be ineffective. Alternatively, our data directly indicate that PKR may also function in the actual induction of IFN itself, possibly by activating NF-κB-dependent transcription of IFN following viral infection. A number of studies have shown that fibrobasts lacking either PKR or the β subunit of IκB kinase (IKKβ) are defective in both dsRNA and VSV-mediated induction of IFN (Yang et al., 1995; Kumar et al., 1997; Chu et al., 1999). In addition, phosphorylation of STAT1 on serine 727 and transactivation has also been reported to be impaired in PKR null cells (Koromilas et al., 1992; Ramana et al., 2000). Defective STAT1 signaling in $PKR^{-/-}$ cells would presumably not only impede the production of STAT1-dependent genes required for the augmentation of IFN production, such as interferon-regulatory factor (IRF) 7, but also critical ISGF3-dependent antiviral

genes required for complete protection of the cell (Marie et al., 1998). Further, the dependence on both the translational and transcriptional activities of PKR for the timely and maximal induction of IFN explain why STAT1 and IFN-α/β-deficient systems are susceptible to virus infection even though PKR is present and why $PKR^{-/-}$ cells and animals are compromised in their defense against VSV even though other components of the IFN system are present.

While IFN was able to rescue $PKR^{-/-}$ EFs from the effects of VSV, it was unable to protect $PKR^{-/-}$ mice following i.n. infection with VSV. One explanation for this could be that cells comprising the pulmonary tissue are not particularly responsive to IFN, though our preliminary investigations indicate IFN induction per se is not impaired (data not shown). Although the highest dose of IFN used in our experiments might not have been sufficient to completely prevent VSV replication, our data is supported by recent evidence showing poor IFN-α/β production and IFN-induced gene expression in human alveolar epithelial and fetal lung cells (Ronni et al., 1997). Only a modest response to IFN can be observed in the lungs of VSV-infected $PKR^{-/-}$ mice, as shown by a two-log reduction in viral titers after IFN treatment. That other groups have shown some protective effects of IFN, when administered i.n. in high doses in mice presumably containing PKR, may again indicate a role for PKR in efficient IFN signaling (Gresser et al., 1975; Heremans et al., 1980; Wyde et al., 1984; Ramana et al., 2000).

It is further interesting that while STAT1- and IFN-α/β receptor-deficient mice are susceptible to lethal VSV infection irrespective of the route of administration, PKR-deficient mice seem to be compromised in their immunity to this virus primarily when it is administered i.n. but not i.v. Thus, PKR seems to be particularly important in preventing productive infection of the respiratory tract and functions directly by inhibiting translation and indirectly by recruiting other components of the immune system to the site of infection (as highlighted by the markedly greater infiltration of leukocytes in lungs from VSV-infected $PKR^{+/+}$ but not $PKR^{-/-}$ mice). In light of these observations, it is noteworthy that pulmonary tissue, while relatively refractory to IFN, contains significantly higher basal levels of PKR than other tissues (Krust et al., 1982).

While the IFN system (including PKR) is clearly critical in protection against VSV infection, an important role for natural neutralizing antibodies, predominantly generated by peritoneal $CD5^+$ B-1 cells, has recently been shown (Ochsenbein et al., 1999). These antibodies represent a spontaneous repertoire of circulating immunoglobulins that play an important role in preventing pathogen, including VSV, dissemination to vital organs in the host (Ochsenbein et al., 1999). Mice lacking B cells but not T cells succumb to lethal VSV infection after i.n. inoculation within 1 week (G. N. B. and S. B., unpublished data) or after i.v. inoculation, as shown elsewhere (Thomsen et al., 1997). Thus, mice lacking an ability to produce antibody, or with a defective IFN response pathway, or lacking PKR (as described here) are acutely sensitive to VSV. Thus, the IFN system is not in itself sufficient to prevent VSV infection and likely requires both B cells (to produce sufficient neutralizing antibodies against progeny virions and for antigen presentation to T cells) and eventually T cells, primarily to provide the assistance that B cells need to switch isotype and maintain a sustained antibody response (Freer et al., 1994). These studies demonstrate the exquisitely synergistic nature of the acquired and innate immune responses in successfully clearing a viral infection.

Given our findings, it was possible that PKR may be important for preventing the replication of viruses other than VSV. As a start to exploring these possibilities, we show that PKR-deficient animals were about 10- to 100-fold more sensitive to the lethal effects of i.n. WSN infection compared to wild-type mice. Although EFs derived from $PKR^{-/-}$ mice exhibited the same degree of cytopathicity compared to EFs from wild-type mice, the former cells were approximately ten times more permissive to WSN replication (data not shown). Viral titers from the lungs of $PKR^{+/+}$ versus $PKR^{-/-}$ mice similarly showed greater virus progeny yield in the absence of PKR. Unlike VSV, WSN infection of mice also induced prominent apoptosis of the infected cells in vivo. We have previously shown a dependence on the FADD/caspase 8 death signaling pathway in WSN-infected immortalized cells undergoing apoptosis that can be potentiated by IFN (Balachandran et al., 2000). Since specific caspase inhibitors proved toxic to EFs, we could not further delineate the mechanisms of cell death in this study. However, we have previously shown that EFs lacking FADD exhibit resistance to influenza virus-mediated apoptosis, while VSV required the presence of functional Apaf-1 to induce cell death (Balachandran et al., 2000). Influenza virus has also been reported by others to regulate Fas-dependent apoptosis in vitro (Takizawa et al., 1996). In the pulmonary tissue of infected mice, however, we found that WSN infection induces the activation of both caspases 8 and 9, possibly because the activation of caspase 8 in vivo causes the recruitment and cleavage of Bid, which then induces the efflux of cytochrome c from the mitochondrion and triggers Apaf-1-dependent activation of caspase 9 (Li et al., 1997; Luo et al., 1998).

Influenza virus replication in wild-type mice is usually restricted to the respiratory tract, a characteristic thought to be determined, at least in part, by the viral hemagglutinin (HA) surface glycoprotein (Klenk and Garten, 1994). However, a recent report showed systemic and fulminant influenza infection following i.n. inoculation of WSN in mice lacking STAT1 and IFN-α/β receptor. These findings indicate that inhibition of viral replication by IFN may be a major determinant of WSN tissue tropism (Garcia-Sastre et al., 1998). Similar to this phenotype, we also found evidence of WSN replication in the spleen and other organs of WSN-infected $PKR^{-/-}$ mice but not wild-type mice (data not shown). This increased range of tropism may be responsible for the greater sensitivity displayed by the $PKR^{-/-}$ mice to fatal i.n. infection by WSN. The fact that WSN is primarily pneumotropic in wild-type animals further strengthens the argument that the respiratory tract is relatively intransigent to the antiviral effects of IFN.

In light of our findings, it is therefore plausible that PKR contributes toward host defense by not only suppressing the translation of viral RNAs but also by augmenting the production of IFN as well as stimulating the

expression of death receptors such as Fas, employed by cytotoxic T cells in destruction of the virally infected cell (Balachandran et al., 1998). Besides playing a role in innate immunity to virus infection, PKR may thus also facilitate viral clearance by invoking the adaptive immune response.

Experimental Procedures

Mice and Primary EFs

The PKR-deficient mice used in this study have been described previously (Abraham et al., 1999). Control 129/terSv × BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in a pathogen-free environment. Primary murine embryonic fibroblasts (EFs) were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin, and used in early passage for all experiments.

Viral Infections

Plaque-purified VSV (Indiana strain) was used to infect EFs in serum-free DMEM for 30 min at 37°C. Progeny virus yield was determined by standard plaque assay of serially diluted virus suspensions on BHK-21 (for VSV) or in the presence of trypsin on MDCK cells (for influenza virus). Sex- and age-matched mice were infected i.n. with virus in volumes of 10 μl administered equally to either nostril and monitored daily for up to 14 days. Mice were sacrificed upon becoming moribund.

Western Blot Analysis, Immunohistochemistry, and Antibodies

Western blots of protein extracts from $PKR^{+/+}$ and $PKR^{-/-}$ EFs were performed as described (Balachandran et al., 1998). Antibodies directed to phosphorylated and total eIF2α have been reported previously (Balachandran et al., 1998). Antitubulin antibody was a gift of Dr. Harish Joshi (Emory University, Atlanta, GA). Neutralizing sheep polyclonal antibody against mIFN-α/β was purchased from Research Diagnostics (Flanders, NJ). Anti-VSV polyclonal antiserum was generated in rabbits using inactivated VSV as antigen. Immunochemical analysis of VSV antigens in infected tissue was performed using the Metal Enhanced DAB Substrate Kit, according to the manufacturer's instructions (Pierce, Rockford, IL).

Analysis of PKR Phosphorylation and VSV Replication

$PKR^{+/+}$ and $PKR^{-/-}$ EFs (5×10^6) were seeded in 100 mM dishes and treated with or without 1000 U/ml murine fibroblast interferon (mIFN-α/β; Sigma, St. Louis, MI) for 18 hr. Cells were subsequently transfected with poly (I:C) using the Lipofectamine Plus reagent (Life Technologies, Gaithesburg, MD) or infected with VSV and labeled with 150 uCi/ml [^{32}P]orthophosphate (Amersham Pharmacia Biotech) in phosphate-free DMEM for 4 hr. Labeled cells were then lysed in buffer I [20 mM Tris (pH 7.5), 50 mM KCl, 400 mM NaCl, 1% NP-40, 1 mM EDTA, 2 μg/ml aprotinin, 1 mM DTT, 25 mM NaF, phosphatase inhibitor cocktail II (Sigma)] and precipitated with poly (I:C)–agarose (Amersham Pharmacia Biotech) as described (Balachandran et al., 1998). The poly (I:C)–agarose beads were washed extensively with buffer I and bound labeled protein visualized by SDS-10% PAGE. For analysis of VSV protein and mRNA synthesis, 5×10^6 $PKR^{+/+}$ and $PKR^{-/-}$ EFs were seeded into 100 mM dishes, treated with or without mIFN-α/β for 18 hr, infected with VSV at an moi of 10 (protein analysis) or 500 (mRNA analysis), and processed as described previously (Pattnaik et al., 1997).

Cell Viability, Apoptosis, and Caspase Activity Analyses

Viability of EFs was determined by their ability to exclude trypan blue dye. Apoptosis was assayed using an annexin V fluorescein Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). Samples were run on a Becton Dickinson FACScan machine and analyzed using CellQuest software. Caspase activity was determined using the ApoAlert FLICE/caspase 8 Fluorescent Assay Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The caspase 9 substrate zLEHD-AFC was purchased from Enzyme Systems Products (Livermore, CA) and adapted for use with the ApoAlert kit. Samples were excited 400 nm and read at 505 nm on a TD 700 fluorometer (Turner Designs, Sunnyvale, CA). The TUNEL assay was performed using the In Situ Cell Death Detection Kit from Boehringer Mannheim (Indianapolis, IN).

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