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Protein Kinase PKR Catalytic Activity is Required for the PKR-dependent Activation of Mitogen-activated Protein Kinases and Amplification of Interferon Beta Induction following Virus Infection

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Abstract

The protein kinase regulated by RNA (PKR) enhances both activation of mitogen-activated protein kinases and the induction of interferon beta (IFN-β) by measles virus defective in C protein expression (C^{ko}). Here we used complementation of human cell lines stably deficient in PKR (PKR^{kd}) to probe the basis of these PKR-mediated responses. We found that PKR^{kd} HeLa and amnion U cell lines were defective for virus-mediated activation of IFN induction signaling components compared to PKR-sufficient control cells. Complementation of PKR^{kd} cells with wildtype PKR, but not with PKR mutants defective in either catalytic activity or dsRNA binding activity, restored JNK, p38 and ATF-2 phosphorylation and enhanced IFN-β induction following infection. By contrast to mammalian PKR, the Z-DNA binding domain-containing fish homologue of PKR, PKZ, lacked the capacity to enhance C^{ko} virus-mediated IFN-β induction. Furthermore, inhibition of virus growth was observed with C^{ko}-infected PKR^{kd} cells complemented with PKR but not with PKZ.

Keywords

PKR; protein kinase; interferon; innate immunity

INTRODUCTION

Interferons (IFNs) are a cornerstone of the innate antiviral immune response that provides an early line of defense against viral infection. In addition to their antiviral activities, IFNs also affect the growth and development of normal and tumor cells (Borden et al., 2007; Joshi et al., 2009; Platanias, 2005; Samuel, 2001). Type I interferons (IFNs α and β) are induced by viral infection and mediate the establishment of an antiviral state in host cells (Randall and Goodbourn, 2008; Samuel, 2001); they also modulate adaptive in addition to innate immune responses (Borden et al., 2007; Le Bon and Tough, 2002). Cellular sensors involved in IFN production include RIG (retinoic acid inducible gene) I-like receptors (RLRs) and Toll-like

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receptors (TLRs) that recognize foreign nucleic acids generated during infection (Kawai and Akira, 2010; Yoneyama and Fujita, 2010). Both RLR and TLR signaling pathways trigger the activation of interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF- κB), which together with activating transcription factor 2 (ATF-2) and c-jun form an enhanceosome complex that activates IFN-β gene transcription (Panne et al., 2007).

PKR is an IFN-inducible protein kinase that mediates both antiviral and antiproliferative effects of IFNs (Garcia et al., 2006; Samuel, 2001). The PKR protein domain structure includes an N-terminal regulatory domain that binds RNA and a C-terminal kinase catalytic domain. Upon RNA binding, PKR undergoes dimerization and autophosphorylation to generate an active kinase (McCormack et al., 1992; McKenna et al., 2007; Ortega et al., 1996; Pindel and Sadler, 2011; Thomis and Samuel, 1993). The best characterized cellular substrate of PKR is the alpha subunit of translation initiation factor 2 (eIF2α), although additional substrates of PKR have been described including IκB kinase, HIV Tat protein, NFAT factor, MPP4 and B65α subunit of PP2A phosphatase (Pindel and Sadler, 2011; Toth et al., 2006). When activated, PKR catalyzes the phosphorylation of eIF2α on serine 51, a modification that leads to an inhibition of translation (Nallagatla et al., 2011; Pindel and Sadler, 2011; Samuel, 1979, 1993). In addition to altering the translation pattern within cells, PKR also modulates signal transduction responses by interacting with and activating components of signaling pathways in response to cellular stresses including virus infection (Pfaller et al., 2011; Pindel and Sadler, 2011; Samuel, 2001). Among the interacting partners described for PKR are the TRAF family of proteins (Gil et al., 2004). Stress-activated protein kinases, c-jun N-terminal kinase (JNK) and p38, also are implicated in the activation of the innate immune response and cytokine production, through phosphorylation of transcription factors c-jun and ATF2 (Liu et al., 2007; Platanias, 2005). Furthermore, PKR has been shown to modulate the activation of JNK and p38 in response to diverse stimuli including lipopolysaccharide, double-stranded RNA (dsRNA), tumor necrosis factor and viral infection (Goh et al., 2000; Iordanov et al., 2000; Sadler and Williams, 2007; Silva et al., 2004; Takada et al., 2007; Zhang et al., 2009).

Fish, like mammals, encode both IFNs and IFN-inducible gene products (Berg et al., 2009; Liu et al., 2011; Sun et al., 2011; Verrier et al., 2011). Among them, a PKR-like kinase protein identified in fish, designated PKZ, is constitutively expressed at low levels and inducible by viral infection or treatment with IFN or poly (I:C). While the C-terminal kinase domain of PKZ is closely related to the kinase domain of mammalian PKR, instead of repeated dsRNA binding domains within the N-terminal region, PKZ possesses two copies of the nucleic acid binding domain for the left-handed Z-conformation DNA (Z-DNA) similar to the Z-DNA binding domains first identified in the mammalian IFN-inducible adenosine deaminase acting on dsRNA (ADAR1) and the poxvirus E3L protein (Bergan et al., 2008; Herbert et al., 1997; Patterson and Samuel, 1995; Rothenburg et al., 2005; Su et al., 2008).

Measles virus (MV), a member of the family of *Paramyxoviridae*, is a nonsegmented negative-sense single-stranded RNA virus possessing 6 genes in the order 3′-N, P/V/C, M, F, H, L-5′ (Griffin, 2007). The C and V proteins are major virulence factors (Cattaneo et al., 2008; Randall and Goodbourn, 2008) that play roles in circumventing the host immune response by inhibiting IFN production and IFN signaling (Nakatsu et al., 2006; Palosaari et al., 2003; Patterson et al., 2000; Shaffer et al., 2003; Sparrer et al., 2011). Previous work showed that the PKR protein mediates a reduction in viral growth and enhancement of apoptosis in cells infected with C-deficient (C^{ko}) measles virus (Toth et al., 2009). Furthermore, PKR plays a role in amplifying the activation of JNK and p38 signaling and the induction of IFN-β by C^{ko} virus infection (McAllister et al., 2010). In order to gain insight into the mechanism by which PKR modulates activation of stress-activated JNK and

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p38 kinases and the induction of IFN-β in response to MV infection, and to test the requirement for PKR activity, we complemented PKR^{kd} cells with either wildtype or mutant forms of PKR or PKZ. Our results reveal that the catalytic activity of PKR is required for optimal activation of the JNK and p38 kinases and maximal induction of IFN-β following infection with C^{ko} virus. By contrast, the PKZ homolog of PKR found in fish, when expressed in PKR^{kd} mammalian cells, does not complement the PKR-dependent phenotype as measured by increased induction of IFN-β or phosphorylation of JNK following C^{ko} virus infection.

MATERIALS AND METHODS

Cells, viruses, and plasmids

Parental human amnion U cells and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (vol/vol) fetal bovine serum (HyClone), 100 μg/ml of penicillin and 100 units/ml of streptomycin (GIBCO/Invitrogen) as previously described (McAllister et al., 2010; Zhang et al., 2009). HeLa cells and U cells made stably deficient in PKR by an integrated short hairpin silencing RNA interference strategy, designated PKR^{kd} and U^{PKRkd}, respectively, were as previously described as were the drug-resistant knockdown vector controls cells (CON^{kd}) (Zhang et al., 2009; Zhang and Samuel, 2007). Knockdown cells were maintained in medium containing 1 μg/ml puromycin (Sigma).

The recombinant Moraten measles virus vaccine strain, mutated to be deficient in C protein expression (C^{ko}) and to include the gene encoding green fluorescent protein inserted downstream of the viral H gene was generously provided by Roberto Cattaneo (Mayo Clinic, Rochester, MN) (Devaux et al., 2008).

The expression vector pcDNA6 was used for expression of the wildtype (WT) human PKR protein and mutants defective for either catalytic activity (K296R) or dsRNA binding activity (K64E) (McCormack et al., 1994; Thomis and Samuel, 1992). The PKR expression plasmid constructs were mutated at four synonymous sites (designated by underlined italics font) in the shRNA target sequence (GCAGGGAGTAGT*CT*T*A*AA*G*TA) located in the PKR open reading frame to circumvent knockdown by the stably expressed silencing RNA while still preserving the PKR amino acid sequence (Zhang et al., 2009). Plasmids were verified by direct sequence analysis. The expression plasmids for PKZ and the mutant deficient in catalytic activity, K199R (Rothenburg et al., 2005), were generously provided by S. Rothenberg (National Institutes of Health, Bethesda).

Virus infections and plasmid transfections

Viral infections were carried out as previously described (McAllister et al., 2010; Toth et al., 2009), using a 50 percent tissue culture dose per cell multiplicity of infection (MOI) of 1. Briefly, U, U^{PKRkd}, CON^{kd}, and PKR^{kd} cells were seeded into 12- or 6-well plates as indicated. After 24 h, monolayers at 80–90% confluency were washed once with OptiMEM and then infected with C^{ko} MV. After 2 h, the monolayers were washed twice with OptiMEM, and then DMEM containing 5% FBS was added and the cultures maintained until harvest. For complementation tests, HeLa PKR^{kd} cells were transfected with the indicated PKR or PKZ expression vector using FuGENE HD transfection reagent (Roche) according to the manufacturer's protocol. Briefly, plasmid DNA was diluted in OptiMEM to the final concentration of 20 ng/μl, and then the FuGENE reagent was added to the diluted DNA to achieve a ratio of DNA (μg) to FuGENE (μl) of 1:2. The DNA-FuGENE complexes were incubated at room temperature for 20 min before addition to cells containing fresh DMEM with 5% FBS. The amount of DNA used was .5 μg/well (12-well plate) or 1 μg/well

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(6-well plate), adjusted using empty vector as necessary dependent upon the amount of expression construct utilized. The medium was changed between 5–6 h after transfection; cells were infected at 15 h after transfection as described above, or left uninfected. Virus growth assays were carried out as previously described (Toth et al., 2009). Titers of virus preparations were determined on Vero cells according to the Spearman-Kärber method (Devaux et al., 2008; Karber, 1931).

Western immunoblot analysis

At 24 h post-infection unless otherwise noted, extracts were prepared with lysis buffer [20 mM Hepes (pH 7.9), 400 mM NaCl, 1mM DTT, 1mM EDTA, and .5% NP-40] containing 50 mM NaF, 1mM Na_2VO_3, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (vol/vol) protease and phosphotase inhibitor cocktails (Sigma) as previously described (Toth et al., 2009). Protein concentration was determined by the Bradford method. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked in Tris-buffered saline containing 5% (wt/vol) either bovine serum albumin (for detection of phosphoproteins) or non-fat milk (for detection of total proteins) for 1 h at room temperature. Primary antibody incubation was performed overnight at 4°C. Rabbit polyclonal antibodies were used to detect PKR (Santa Cruz Biotechnology), ATF-2 (Santa Cruz Biotechnology), phospho-ATF-2 Thr71 (Cell Signaling Technology), p38 (Santa Cruz Biotechnology), Jun N-terminal kinase (JNK) (Santa Cruz Biotechnology), phospho-JNK Thr183/Tyr185 (Cell Signaling Technology), IRF-3 (Santa Cruz Biotechnology), and GFP (Santa Cruz Biotechnology). Rabbit monoclonal antibodies were used to detect phospho-PKR Thr446 (Epitomics), phospho-IRF-3 Ser396 (Cell Signaling Technology), phospho-p38 Thr180/Tyr182 (Cell Signaling Technology), and phospho-eIF2α Ser51 (Epitomics); mouse monoclonal antibodies were used to detect β-actin (Sigma) and poly (ADP-ribose) polymerase (PARP) (BD Bioscience). Anti-Myc antibody for detection of epitope tagged PKZ was from Roche, and antibody against MV viral H protein was provided by Roberto Cattaneo (Rochester, MN) as previously described (Toth et al., 2009). Western immunoblot detection was performed with IRDye 800CW-conjugated anti-rabbit immunoglobulin G or IRDye 680-conjugated anti-mouse IgG secondary antibody according to the manufacturer's protocols. The immunoreactive bands were quantified using an Odyssey infrared imaging system (Li-COR Biosciences) and obtained values normalized to β-actin as a loading control.

Quantitative real-time PCR

IFN-β transcripts were measured by quantitative real-time PCR (qPCR) as previously described (McAllister et al., 2010). RNA was isolated from uninfected or infected cells 24 h after infection with C^{ko} virus by the RNeasy (Qiagen) protocol. cDNA was prepared by reverse transcription carried out using 1 μg of RNA, random hexamer oligonucleotide primers, and SuperScript II (Invitrogen). qPCR analyses were performed in duplicate with each cDNA template using IQ SYBR Green Supermix (Bio-Rad) and a Bio-Rad MyIQ multicolor real-time qPCR instrument. The forward and reverse primers for IFN-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as described previously (McAllister et al., 2010). For IFN-β transcripts, the qPCR program was set for 3-min hot start followed by 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C, repeated 45 times. IFN-β values were normalized to GAPDH values.

Luciferase reporter assay

PKR^{kd} Hela cells in 12-well plates were transfected with the firefly luciferase construct pGL3 control (Promega) and either an empty vector or indicated PKR or PKZ expression vector using FuGENE HD transfection reagent (Roche). To achieve comparable levels of expression, 0.5 μg of wildtype or 0.1 μg of mutant plasmid DNA construct for PKR or PKZ

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were co-transfected with 0.05 μg of pGL3 control plasmid. At 24 h after transfection cell extracts were prepared and analyzed for luciferase activity according to the manufacturer's protocol (Promega) using an OPTOCOMP I luminometer. Luciferase activity was normalized to total extract protein.

RESULTS

PKR enhances the phosphorylation of MAP kinases and ATF2 following measles virus infection

Prior studies established that the attenuated Moraten vaccine strain MV C^{ko} mutant, deficient for C protein expression, is a robust inducer of IFN-β compared to either WT or V^{ko} virus. The enhanced IFN-inducing capacity of C^{ko} virus is PKR-dependent and correlates with activation of MAP kinases (McAllister et al., 2010). To further define the role and generality of PKR as a mediator of IFN-β induction in MV-infected human cells, the PKR requirement for activation of signaling components following C^{ko} virus infection was examined using two different human cell lines, HeLa and amnion U, made stably deficient in PKR expression (PKR^{kd}) by a shRNA silencing strategy (Zhang et al., 2009; Zhang and Samuel, 2007). The HeLa PKR^{kd} cells contain less than 5% of the PKR protein found in PKR-sufficient control HeLa cells (CON^{kd}); the U PKR knockdown cells (U^{PKRkd}) contain less than 10% of the PKR protein expressed in parental U cells (U) (Fig. 1).

As shown by the Figure 1A quantifications of immunoblots, following C^{ko} virus infection, the phosphorylation of JNK, p38 and ATF2 were impaired in PKR^{kd} HeLa cells compared to control (CON^{kd}) cells. The MAPK pathway activations seen in PKR-sufficient CON^{kd} HeLa cells correlated with enhanced phosphorylation of both PKR and eIF2α as shown by representative immunoblots (Fig. 1B). By contrast, IRF3 phosphorylation was not impaired in PKR deficient (PKR^{kd}) cells compared to the PKR sufficient CON^{kd} cells following infection. Quantitation of the western blots revealed that the level of phosphorylated JNK, p38 and ATF2 was increased ~5 to 10-fold by infection of CON^{kd} HeLa cells, and to higher levels in the PKR-sufficient CON^{kd} compared to PKR-deficient PKR^{kd} HeLa cells (Fig. 1A).

As a test of the generality of the results observed with PKR^{kd} HeLa cells, we assessed the PKR dependency of C^{ko} virus-induced activation of MAPK signaling components in a second human cell line, amnion U cells, in which PKR also had been stably knocked down using the shRNAi strategy. PKR^{kd} U cells showed results similar to those seen with PKR^{kd} HeLa cells. The phosphorylations of JNK, p38 and ATF2 were enhanced in C^{ko}-infected PKR-sufficient U cells compared to PKR-deficient U ^{PKRkd} cells at 24 h after infection, and compared to uninfected cells or cells infected for only 6 h (Fig. 1C). Activation of PKR and increased phosphorylation of eIF2α likewise were increased at 24 h postinfection in PKR sufficient U cells compared to U^{PKRkd} cells (Fig. 1C), similar to the observations with HeLa cells (Fig. 1B).

Viral protein expression was assessed by determining the levels of two proteins: GFP, a reporter engineered downstream of the viral H gene for expression by recombinant virus; and, the viral H glycoprotein. Inhibition of GFP (Fig. 1A,C) and H (Fig. 1A) protein expression were seen in CON^{kd} and U parental cells, and this inhibition correlated with an increased activation of PKR and phosphorylation of eIF2α in these PKR-sufficient cells (Fig. 1B,C). Knockdown of PKR led to increased viral protein production. Western blot quantitation revealed that the expression levels of GFP and H were ~3-fold higher in the PKR deficient compared to PKR sufficient cell lines (Fig. 1A,C).

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Catalytic and RNA binding activities of PKR are required for the PKR-mediated enhancement of MAPK signaling and increased PARP cleavage following C^{ko} infection

To test the requirement for PKR catalytic and RNA-binding activities in conferring the PKR dependency seen for MAP kinase and ATF2 phosphorylation in C^{ko}-infected cells, PKR^{kd} HeLa cells were complemented with either a catalytically inactive mutant PKR (K296R), an RNA-binding mutant (K64E), or WT PKR. To prevent knockdown of the ectopically expressed PKR by the stably expressed shRNA, the PKR cDNA sequence was mutated at the RNAi target site as described under Materials and Methods in a manner that circumvented knockdown without altering the amino acid sequence of the encoded PKR protein. Complemented cells were either left uninfected, or were infected with the C^{ko} virus. Because K296R and K64E mutant proteins are expressed more efficiently than WT PKR protein due to PKR autoregulatory effects seen in transfected cells (Barber et al., 1993; McCormack et al., 1994; Thomis and Samuel, 1992), different amounts of plasmid DNA were used in the transfections to achieve comparable PKR expression levels of the WT, K296R and K64E proteins as measured by western blot analysis (Fig. 2, *left*, lanes 3–8).

For PKR^{kd} HeLa cells transfected with empty vector (Vec), no PKR phosphorylation was observed and only modest increases in phosphorylation of JNK, p38 and ATF2 were seen following C^{ko} virus infection (Fig. 2, *right*, lanes 1,2). Complementation of PKR^{kd} cells by expression of WT PKR protein increased the phosphorylation of JNK, p38 and ATF2 following infection (Fig. 2, *right*, lane 4) compared to PKR^{kd} cells complemented with either the K296R or K64E mutant (Fig. 2, *right*, lanes 6,8). The PKR-mediated activation of MAP kinase and ATF2 in C^{ko}-infected cells correlated with the phosphorylation of PKR and eIF2α (Fig. 2, *left*, lane 4). Furthermore, viral protein expression measured by GFP was inhibited in cells complemented with WT PKR, but in contrast, was increased in the K296R and K64E complemented cells following infection. The level of PARP cleavage, as an indicator of apoptosis, was elevated in cells expressing WT PKR but not in cells expressing either the catalytic or RNA-binding mutant protein. The increase in level of phosphorylated PKR in transfected cells observed with WT PKR in the absence of infection (Fig. 2) most likely is due to the plasmid transfection process that can give rise to activating dsRNA (Chiu et al., 2009; Wang and Samuel, 2009).

PKZ does not restore MAP kinase activation in PKR^{kd} cells infected with C^{ko} virus

Phylogenetic analyses reveal that the kinase domain of PKZ from fish is closely related to that of mammalian PKR, but PKZ possesses two Z-DNA binding domains in the N-terminal region instead of the two RNA-binding domains found in PKR (Rothenburg et al., 2005). To test whether PKZ is able to restore MAP kinase activity in the PKR-deficient HeLa cells, WT PKZ was expressed in the PKR^{kd} cells and activation of JNK was assessed following virus infection. Neither the expression of WT PKZ nor a catalytic mutant (K199R) form of PKZ increased JNK phosphorylation in C^{ko}-infected cells (Fig. 3, lanes 6,8). By contrast, as a positive control, the expression of WT PKR rescued the phosphorylation of JNK relative to vector-transfected cells following C^{ko} infection (Fig. 3, lanes 2,4). While PKZ did not restore MAP kinase activation in the PKR^{kd} cells infected with C^{ko} virus (Fig. 3), the WT PKZ construct like the WT PKR construct inhibited expression of the luciferase reporter when expressed in PKR^{kd} cells, whereas neither the K199R PKZ mutant or K296R PKR mutant were inhibitory in the cotransfection reporter assay (Fig. 4). Finally, we also assessed the effect of PKZ on induction of apoptosis following infection. As shown in Figure 3, PARP cleavage was not detectably enhanced following C^{ko} infection in cells expressing PKZ (Fig. 3, lane 6,8), but was increased in cells expressing PKR (Fig. 3, lane 4).

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Expression of PKR but not PKZ in PKR^{kd} cells reduces the virus-coded GFP reporter expression

We earlier found that the knockdown of PKR rescued the growth of C^{ko} mutant virus (Toth et al., 2009). Consistent with this finding, we observed herein that WT PKR reduced the growth of C^{ko} virus in complemented PKR^{kd} cells as measured by GFP expression (Fig. 2). We next examined virus growth in PKR^{kd} HeLa cells expressing PKZ compared to PKR (Fig. 5). The GFP signal measured by fluorescence was reduced in PKR^{kd} cells complemented with PKR relative to that of vector-transfected cells (Fig. 5A). However, the signal intensity of GFP expression was similar between vector and PKZ-transfected cells. Somewhat increased GFP expression was seen in cells expressing the K296R mutant form of PKR, an effect possibly due to a dominant negative activity of the ectopically expressed catalytic mutant on the residual endogenous WT PKR protein (Fig. 5A). Western blot analysis of GFP protein expression (Fig. 5B) was in general agreement with the results observed by fluorescence (Fig. 5A), suggesting that PKR but not PKZ was able to mediate impairment of C^{ko} mutant virus growth. Similar to the observation for the engineered GFP reporter protein, viral H protein expression was also impaired in PKR^{kd} cells complemented with WT PKR but not with the K296R mutant PKR or WT PKZ (Fig. 4B). As controls, western immunoblot analysis with antibody against β-actin (Fig. 5B) and phase-contrast images (data not shown) indicated comparable cell numbers for the different complemented cell cultures that were analyzed.

As a direct measure of the effect of PKR or PKZ complementation on C^{ko} virus replication in PKR^{kd} cells, virus yields were determined at 24 h after infection at an MOI of 1 by the 50% $TCID_{50}$ titration assay on Vero cells. The yields obtained for PKR^{kd} cells complemented with vector (4.8×10^5 $TCID_{50}$/ml), WT PKZ (2.7×10^5) or K296R mutant PKR (6.3×10^5) were comparable, whereas the yield for WT PKR-complemented cells (6.0×10^4) was nearly 1 $\log_{10}$ lower.

Wildtype PKR, but not PKZ, enhances IFN-β induction by C^{ko} measles virus

Next we compared the ability of PKR and PKZ to restore the IFN-β induction phenotype in PKR^{kd} cells following infection with C^{ko} virus. Either WT or the catalytic mutant of PKR, or PKZ, was expressed in PKR^{kd} HeLa cells and the level of IFN-β RNA was measured by qPCR at 24 h after C^{ko} virus infection. As shown in Figure 6, complementation of PKR^{kd} cells with WT PKR enhanced the induction of IFN-β ~6-fold relative to either the vector control cells or cells complemented with the K296R mutant following infection (Fig. 6A). No amplification of IFN-β expression was observed in the PKR^{kd} cells expressing PKZ, either WT or the K199R mutant (Fig. 6A,B). These results suggest that PKR catalytic activity is required to enhance the induction of IFN-β in MV-infected cells, and that the PKR requirement cannot be fulfilled by PKZ.

DISCUSSION

The host response to viral infection includes the activation of signal transduction networks that lead to changes in gene expression in infected cells, as illustrated by the innate antiviral immune response (Kawai and Akira, 2010; Yoneyama and Fujita, 2010). Among the cellular proteins implicated in modulating virus-induced responses, including activation of stress-associated JNK and p38 MAP kinases and induction of interferon, is the RNA-dependent protein kinase PKR (Garcia et al., 2006; Pfaller et al., 2011; Pindel and Sadler, 2011; Samuel, 2001). The importance of PKR in the host response is illustrated by recent findings with measles virus. For MV, which has tropism for human cells, the virus-host interplay is affected by virulence factors encoded by the viral P/V/C gene (Cattaneo et al., 2008; Griffin, 2007; Randall and Goodbourn, 2008). Recombinant mutant virus deficient for C protein

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expression grows poorly relative to wildtype parental virus; a major effector of the C phenotype is the PKR kinase (Toth et al., 2009). C^{ko} mutant virus is a robust inducer of IFN-β and a potent activator of the JNK and p38 stress-activated kinases, both of which are PKR-dependent responses (McAllister et al., 2010). What has been less clear is the mechanism by which PKR acts to affect signaling and induction of IFN-β. Does PKR act as an enzyme, or as an RNA-binding protein, or as both when mediating signaling effects (Garcia et al., 2006; Pfaller et al., 2011)?

Here we present evidence obtained through complementation analyses that PKR plays a key role in triggering activation of JNK and p38 and amplifying the induction of IFN-β in measles virus infected cells in a manner that is dependent upon PKR catalytic activity. Ectopic expression of wildtype PKR restored MAPK activation and IFN-β induction in PKR-deficient human cells. By contrast, neither catalytically deficient PKR nor RNA binding deficient PKR nor the related PKZ kinase was able to complement PKR-deficient human cells to rescue PKR-dependent signaling phenotypes. Several important points emerge from these findings.

First, we demonstrate using two types of human cell lines, HeLa and amnion U, the generality of the finding that the stable knockdown of PKR leads to an impaired phosphorylation of JNK and p38 as well as ATF2 following infection with C^{ko} mutant measles virus. While the C^{ko} virus also was a robust inducer of IRF-3 phosphorylation, this IRF3 response was independent of PKR both in HeLa and U cells infected with MV. Our results obtained with the two human cell lines demonstrating PKR dependency for MAPK activation in C^{ko} virus-infected cells are consistent with and extend earlier findings obtained with the HeLa PKR^{kd} cells infected with C^{ko} measles virus (McAllister et al., 2010) or ΔE3L mutant vaccinia virus (Zhang et al., 2009).

What is the mechanism by which PKR mediates activation of JNK and p38 MAP kinases? Likewise, what is the mechanistic basis of the PKR-mediated amplification of IFN-β induction observed with C^{ko} measles virus (McAllister et al., 2010)? Among the ways by which PKR might enhance JNK and p38 phosphorylation and amplify IFN production are those that would include a catalytic-dependent enzymatic function for PKR, or alternatively, those that might require the PKR protein only in a catalytic-independent role perhaps as an adaptor or scaffold protein rather than or in addition to a role as an enzyme. Our results argue against simply an adaptor or scaffold role for the PKR protein that does not also involve PKR as an enzyme. The K296R mutant of PKR, that lacks catalytic activity but binds dsRNA normally (McCormack and Samuel, 1995; Thomis and Samuel, 1992; Toth et al., 2006), was not able to complement the PKR^{kd} cells to restore either the activation of MAP kinases or the induction of IFN-β following C^{ko} infection. While the observed phenotype of C-protein deficient virus characterized by enhanced activation of PKR and subsequent PKR-dependent signaling responses might implicate C protein as an antagonist of PKR, the inability of Sendai virus C protein to impair PKR activation by Newcastle disease virus (Takeuchi et al., 2008) and the inability of MV C protein to impair PKR activation by vaccinia virus deficient for E3L protein expression (Toth et al., 2009), together with the inability to demonstrate interaction between PKR and MV C proteins (Toth et al., 2009), suggest that C protein does not inhibit PKR activation directly. The alternative possibility, that paramyxovirus C proteins are indirect inhibitors of PKR activation by limiting production of aberrant dsRNAs that activate PKR, seems more likely (Boonyaratanakornkit et al., 2011; Takeuchi et al., 2008; Toth et al., 2009). Interestingly, wild-type MV displays a PKR activation phenotype similar to C^{ko} MV in cells deficient for adenosine deaminase acting on RNA (ADAR1), an editing enzyme that destabilizes dsRNA structure through deamination (Li et al., 2012).

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The finding that PKR is required for maximal induction of IFN-β is not limited to measles virus-infected (McAllister et al., 2010) or dsRNA-transfected (McAllister and Samuel, 2009) cells. PKR also is necessary for maximal production of IFN-β in cells infected with other RNA viruses, both positive-stranded and double-stranded, including West Nile virus, encephalomyocarditis virus, Semliki Forest virus and rotavirus (Schulz et al., 2010; Sen et al., 2011). However, the basis of the PKR effect in the different virus-cell systems is not sufficiently well defined to conclude whether the biochemical mechanism is possibly qualitatively similar for the different viruses, or whether PKR is affecting the host response to viral infection differently for different viruses (Pfaller et al., 2011).

Given the requirement for catalytic activity, it is tempting to speculate that the amplification of IFN-β expression mediated by PKR is in part a translational effect through eIF-2α phosphorylation or phosphorylation of additional substrates in manner whereby the functional consequence is displayed at the level of translation, for example phosphorylation of IκB kinase by PKR which has been described (Zamanian-Daryoush et al., 2000). We find that PKR catalytic activity is required for rescue of IFN-β induction and MAPK phosphorylation in the HeLa PKR^{kd} cells following C^{ko} infection, which would be consistent with this notion. Furthermore, activation of JNK, p38 and ATF2 phosphorylation in a PKR-dependent manner in the HeLa and U cell lines correlated with PKR autophosphorylation on Thr446, an increase in eIF-2α phosphorylation and the inhibition of viral protein expression.

PKZ, a PKR-like kinase present in fish, was unable to rescue the phosphorylation of JNK or restore IFN-β induction as was seen for PKR when expressed in the HeLa PKR^{kd} cells. PKZ has been reported to phosphorylate eIF2α in vitro and to inhibit reporter expression in transfected mammalian cells (Bergan et al., 2008; Rothenburg et al., 2005, 2008). We confirmed that PKZ inhibits reporter gene expression in transfected cells, however the mechanism of activation of PKZ, unlike PKR, is not defined. Also, it is unknown whether PKR and PKZ display similar substrate specificity beyond eIF2α. The fact that both PKR and PKZ affect translation by a mechanism that reportedly involves eIF2α phosphorylation (Bergan et al., 2008; Rothenburg et al., 2008), but that only PKR and not PKZ is able to complement the signaling defect in the PKR^{kd} cells, is consistent with a dual function for PKR, acting both as an enzyme and as a scaffold adaptor protein. Activation of NFκB and IFN-β induction by PKR is dependent upon the IPS-1 adaptor (McAllister et al., 2010) and TRAFs (Gil et al., 2004). Colocalization and physical interaction between PKR and TRAF family proteins has been described, in which the K296R mutant form of PKR or the C-terminal PKR kinase alone, is sufficient for interaction at least with TRAF5 (Gil et al., 2004). However, others have concluded that the ability of PKR to mediate NFκB activation resides in the N-terminal region of PKR and requires both dsRNA binding domains (Bonnet et al., 2006).

Taken together, our results firmly establish that PKR catalytic activity is required for amplification of IFN-β induction and enhancement of MAP kinase activation in C^{ko} measles virus infected cells, and that the PKR requirement can not be fulfilled by PKZ. Our results furthermore suggest the intriguing possibility that PKR functions both as an enzyme and a scaffold protein in modulating signaling events in measles virus C^{ko} infected cells. Although PKZ is related to PKR, PKZ seemingly is unable to complement the functional roles of PKR, as an enzyme or as an interacting protein *per se*.

Acknowledgments

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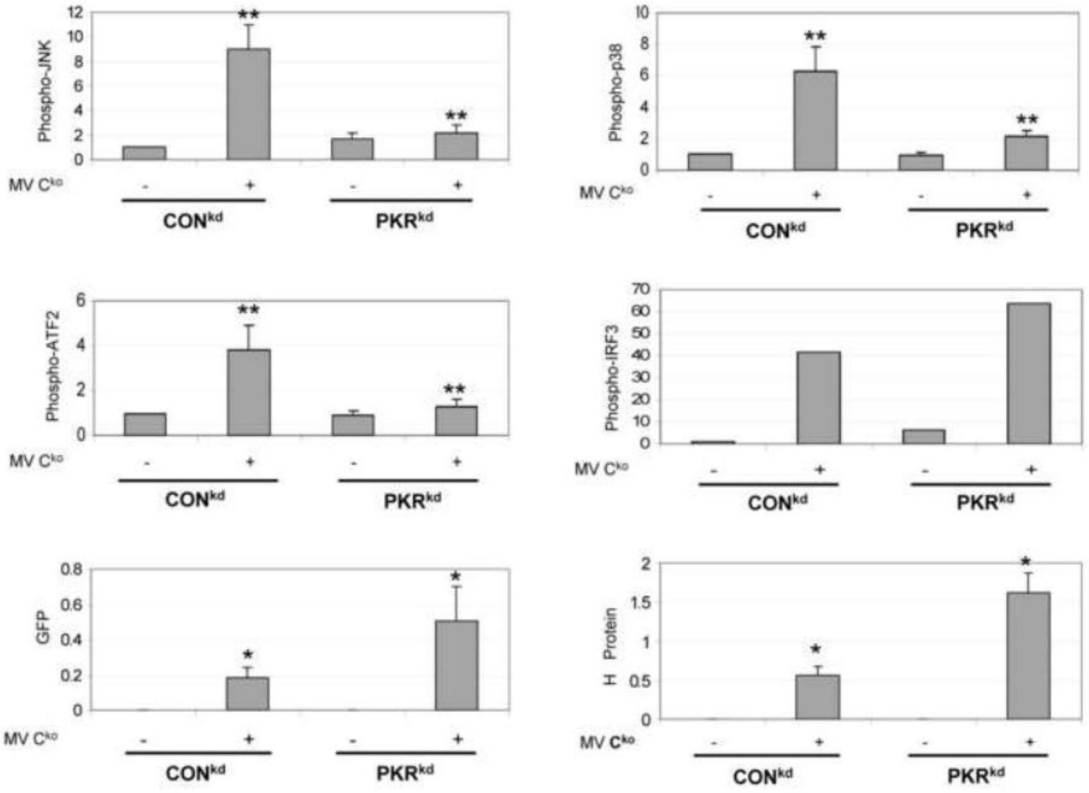

Figure 1A

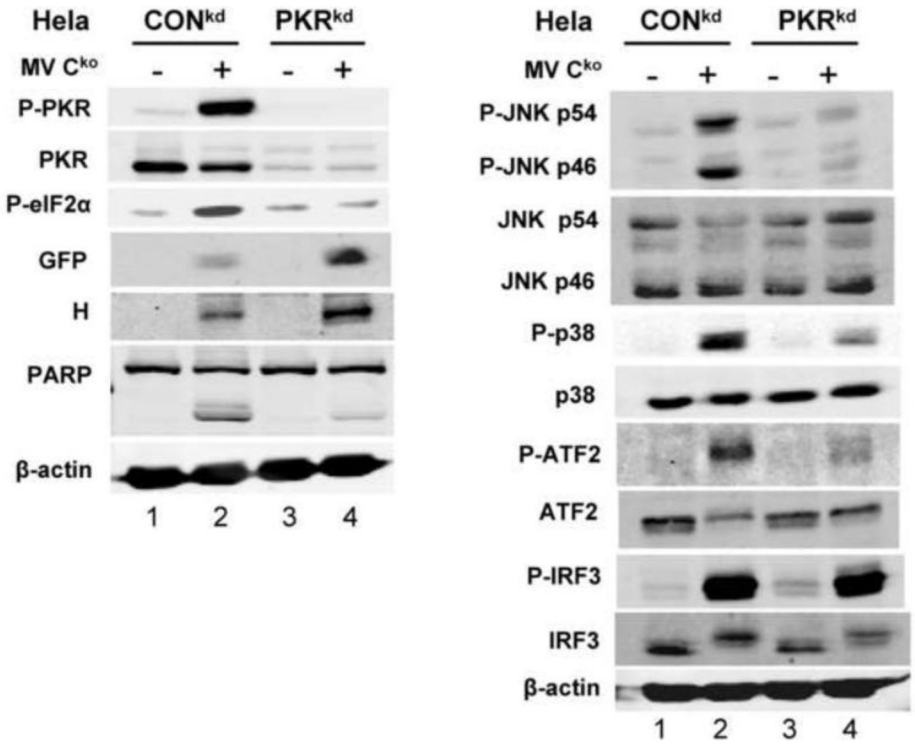

Figure 1B

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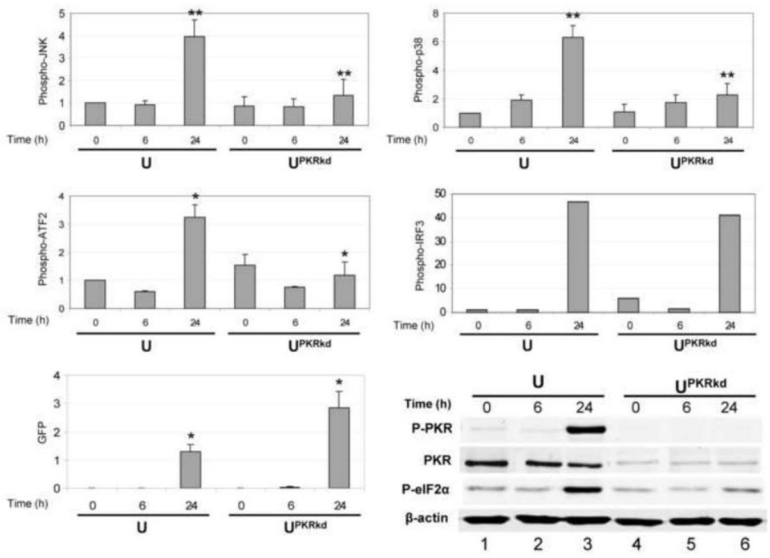

Figure 1C

FIGURE 1. Activation of JNK, p38 and ATF2 phosphorylation is PKR dependent, but IRF3 phosphorylation is PKR independent, in C^{ko}-infected cells

(A) Whole-cell extracts were prepared from either uninfected (−) or infected (+) HeLa cells (CON^{kd} or PKR^{kd}) at 24 h after infection with C^{ko} measles virus. Western blot analyses were performed and the blots quantified for levels of phosphorylation of JNK, p38, ATF2 and IRF3, and GFP and H protein expression, by infrared imaging as described under Materials and Methods. (B) Representative blots are also shown for PKR, phospho-PKR (P-PKR), and phospho-eIF2α (P-eIF2α), in addition to the proteins quantified in (A) above. (C) Quantitation of MAP kinase, ATF2 and IRF3 phosphorylation and GFP expression determined by immunoblot analyses of extracts prepared from amnion U cells (U) or a stable PKR knockdown clone (U^{PKRkd}) infected for 6 or 24 h with C^{ko} virus (6, 24), or left uninfected (0). Quantifications were performed as for Hela cells shown in (A). Representative western immunoblots for PKR, phospho-PKR (P-PKR), and phospho-eIF2α (P-eIF2α) are shown for extracts from amnion U cells, uninfected (0) or infected (6, 24 h) with C^{ko} virus. The results shown in (A) and (C) are the means with standard deviation (n= 3). *, P< 0.05; * *, P< 0.01.

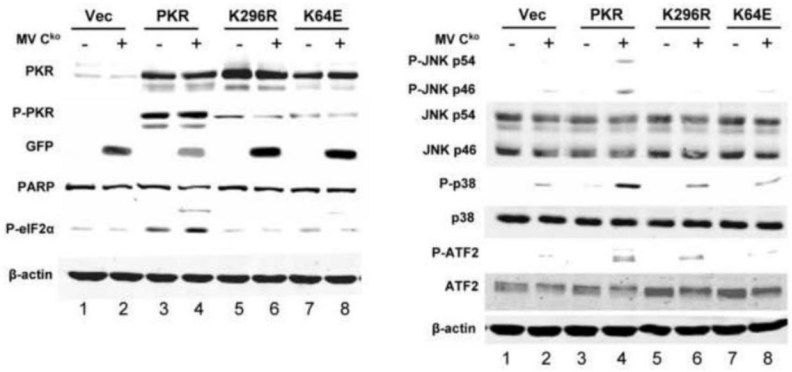

FIGURE 2. Expression of wildtype PKR protein in PKR^{kd} cells rescues JNK, p38 and ATF-2 phosphorylation and enhances PARP cleavage following MV C^{ko} infection

PKR^{kd} HeLa cells were transfected with either an empty vector (Vec) or the expression construct encoding wildtype (WT), catalytic mutant (K296R) or RNA-binding mutant (K64E) PKR. At 15 h after transfection, cells were either mock infected (−) or infected with MV C^{ko} (+) for 24 h. Western immunoblot analyses were performed on whole-cell extracts using antibodies against phospho-PKR (P-PKR), PKR, phospho-eIF2α (P-eIF2α), GFP and PARP (*left panel*); and, phospho-JNK (P-JNK), JNK, phospho-p38 (P-p38), p38, phospho-ATF2 (P-ATF2), and ATF2 (*right panel*). β–actin, loading control.

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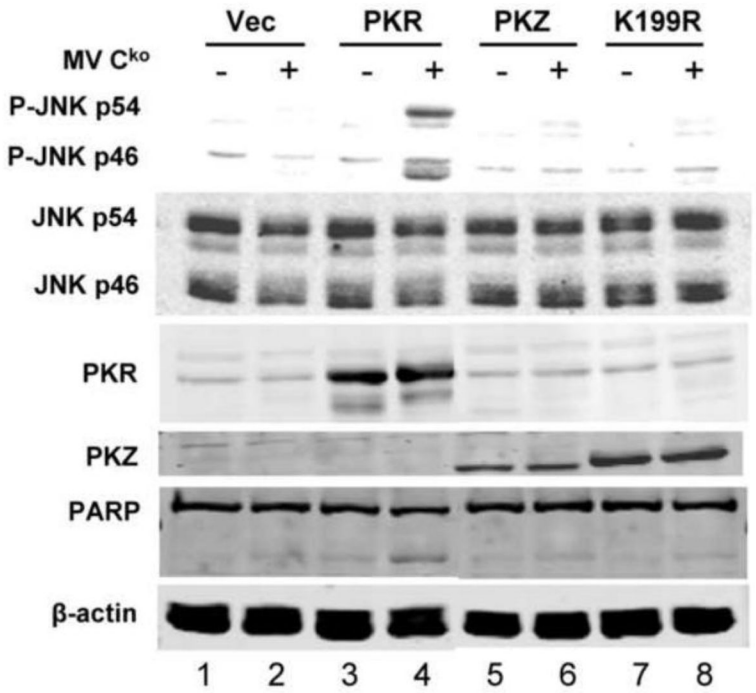

FIGURE 3. Expression of PKZ in PKR^{kd} cells does not rescue JNK phosphorylation or increase PARP cleavage following MV C^{ko} infection

PKR^{kd} HeLa cells were transfected with either empty vector (Vec) or the expression construct encoding wildtype PKR (PKR), wildtype PKZ (PKZ) or the PKZ catalytic mutant (K199R). After transfection for 15 h, cells then were either mock infected (−) or infected with MV C^{ko} (+) for 24 h. Western immunoblot analyses were performed on whole-cell extracts using antibodies against phospho-JNK (P-JNK), JNK, PKR, Myc for Myc-tagged-PKZ (PKZ) and PARP. β–actin, loading control.

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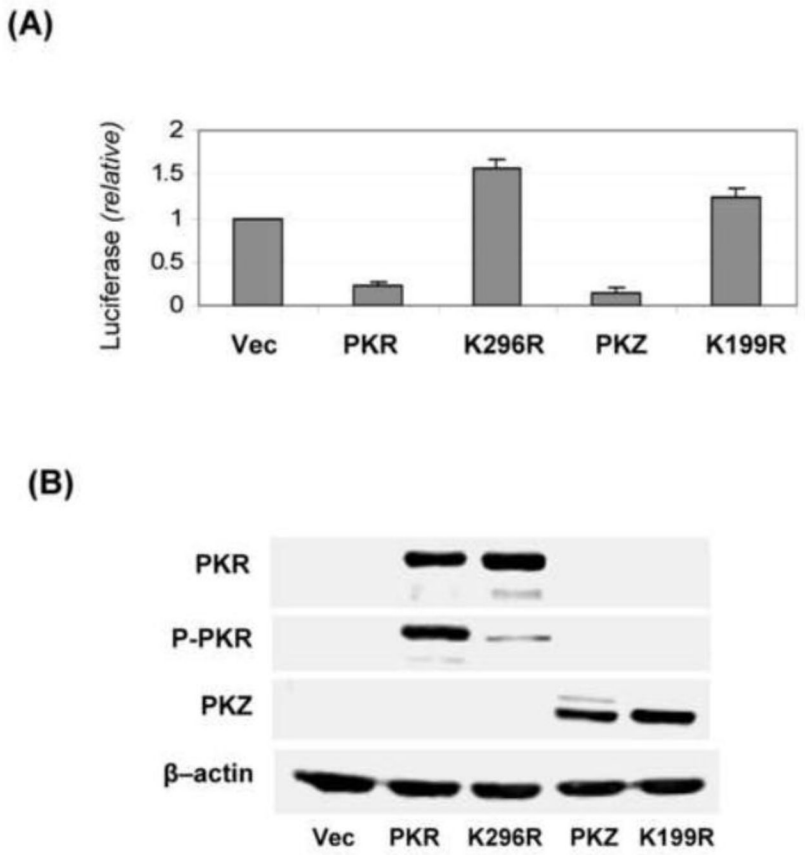

FIGURE 4. Expression of either PKR or PKZ in PKR^{kd} cells inhibits luciferase reporter synthesis

PKR^{kd} Hela cells were co-transfected with the pGL3 control plasmid and either an empty vector (Vec) or the expression construct encoding wildtype PKR (PKR), wildtype PKZ (PKZ), PKR catalytic mutant (K296R), or the PKZ catalytic mutant (K199R). At 24 h after transfection whole-cell extracts were prepared and analyzed. (A) Luciferase activity was measured as described under Material and Methods. The means and standard deviations determined from three independent experiments. (B) Representative western immunoblots obtained with whole-cell extracts using antibodies against phospho-PKR (P-PKR), PKR, and Myc for Myc-tagged-PKZ (PKZ); β–actin, loading control.

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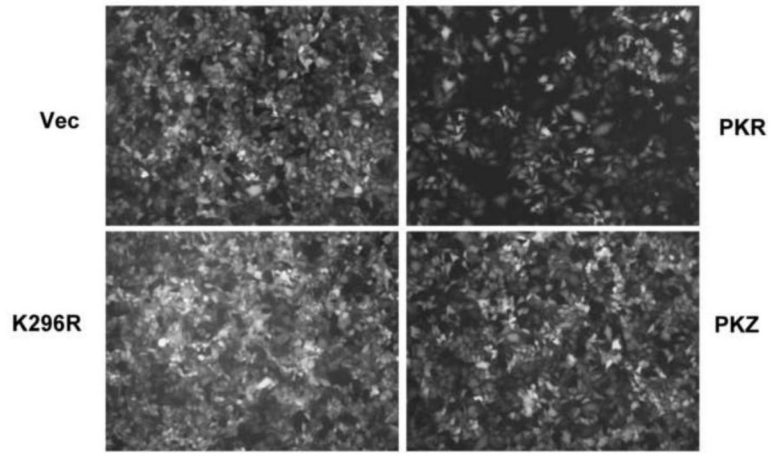

Figure 5A

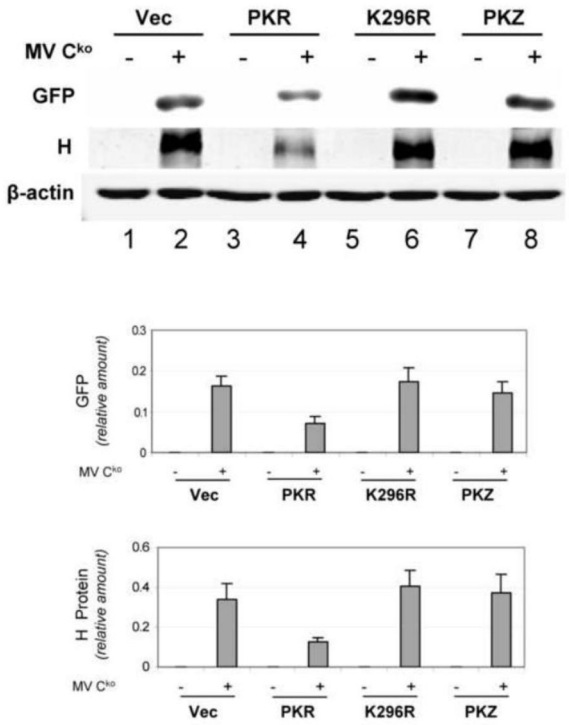

Figure 5B

FIGURE 5. Expression of PKR but not PKZ impairs C^{ko} mutant measles virus growth in PKR^{kd} cells

PKR^{kd} Hela cells were transfected with an empty vector (Vec) or the expression construct encoding wildtype PKR (PKR), the PKR catalytic mutant (K296R), or wildtype PKZ as indicated. At 15 h after transfection cells were either mock infected (−) or infected with MV C^{ko} (+). (A) Fluorescence images taken at 24 h after infection. (B) Representative western immunoblot analyses of complemented PKR^{kd} HeLa whole-cell extracts prepared 24 h after infection, using antibodies against MV encoded proteins (GFP and H) and β-actin (*upper*). Quantitation of GFP and H protein expression in complemented PKR^{kd} HeLa cells at 24 h after infection (*lower*).

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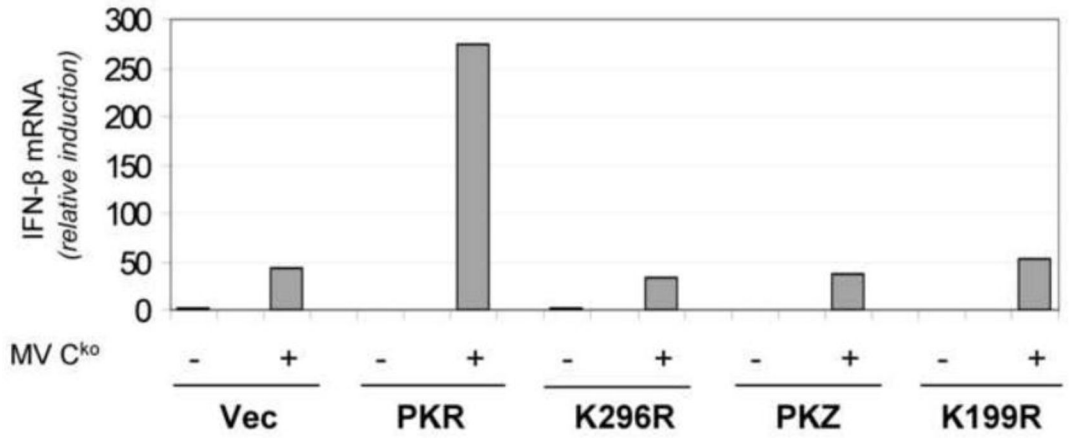

Figure 6A

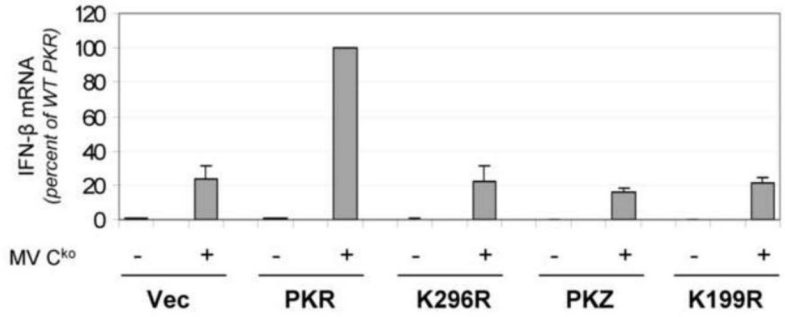

Figure 6B

FIGURE 6. Expression of wildtype PKR but not PKZ enhances IFN-β induction by measles virus

PKR^{kd} HeLa cells were transfected with the indicated plasmids and at 15 h after transfection were either mock infected (−) or infected (+) with C^{ko} virus. At 24 h after infection total RNA was prepared and IFN-β transcript levels normalized to GAPDH were determined using quantitative real-time PCR. (A) Representative induction results shown for one of three independent experiments normalized to vector-transfected, uninfected cells. (B) The mean induction of IFN-β mRNA and standard deviations determined for three independent experiments. The results are normalized to C^{ko}-infected, transfected cells expressing WT PKR taken as 100%.