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West Nile virus infection does not induce PKR activation in rodent cells

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ABSTRACT

dsRNA-activated protein kinase (PKR) is activated by viral dsRNAs and phosphorylates eIF2a reducing translation of host and viral mRNA. Although infection with a chimeric West Nile virus (WNV) efficiently induced PKR and eIF2a phosphorylation, infections with natural lineage 1 or 2 strains did not. Investigation of the mechanism of suppression showed that among the cellular PKR inhibitor proteins tested, only Nck, known to interact with inactive PKR, colocalized and co-immunoprecipitated with PKR in WNV-infected cells and PKR phosphorylation did not increase in infected Nck1,2−/− cells. Several WNV stem-loop RNAs efficiently activated PKR *in vitro* but not in infected cells. WNV infection did not interfere with intracellular PKR activation by poly(I:C) and similar virus yields were produced by control and PKR−/− cells. The results indicate that PKR phosphorylation is not actively suppressed in WNV-infected cells but that PKR is not activated by the viral dsRNA in infected cells.

Introduction

PKR is a serine/threonine kinase composed of an N-terminal regulatory domain that contains two dsRNA binding motifs (DRBMs) and a C-terminal kinase domain (Meurs et al., 1990; Nanduri et al., 1998). These domains are connected by a spacer that provides an interface for dimerization (McKenna et al., 2007). It has been proposed that in the unphosphorylated state, the N-terminal regulatory domain interacts with the C-terminal catalytic domain to inhibit kinase activity (Nanduri et al., 2000). Activation of PKR by dsRNA results in the formation of dimers that are stabilized by autophosphorylation at multiple residues, including Thr446 and Thr451 that are located within the activation loop of the kinase domain and essential for PKR activation (Romano et al., 1998). To date, 18 PKR phosphorylation sites have been identified. Most are serine residues but some are threonine or tyrosine residues (Su et al., 2006; Toth et al., 2006). Active PKR dimers eject the activating dsRNA, presumably, due to phosphorylation of N-terminal residues and then phosphorylate eIF2a (Jammi and Beal, 2001). PKR is constitutively and ubiquitously expressed at low levels due to a kinase conserved sequence (KCS) site in its promoter (Toth et al., 2006). PKR expression is upregulated by Type I IFN which can be produced in response to a viral infection. The majority of PKR is located in the cytoplasm where a portion is associated with ribosomes. Some of the PKR in the nucleus associates with nucleoli (MacQuillan et al., 2009; Tanaka and Samuel, 1994; Toth et al., 2006).

A ternary complex consisting of GTP-eIF2 and a methionyl-tRNA delivers the charged initiator tRNA to the 40S ribosomal subunit of the 43S preinitiation complex but translation initiation requires the hydrolysis of the eIF2-bound GTP to a GDP (Hershey, 1991; Majumdar and Maitra, 2005). Under stress conditions, the alpha-subunit of eIF2 is phosphorylated by one of four eIF2a kinases: general control non-repressed 2 (GCN2), heme-regulated inhibitor (HRI), PKR-like endoplasmic reticulum (ER) kinase (PERK), or PKR (Kaufman, 1999). The eIF2a kinases share a conserved kinase domain that mediates eIF2a phosphorylation, but each responds to a different stress due to its unique regulatory domain (Kaufman, 1999). Phosphorylation of eIF2a on Ser51 leads to the formation of a high-affinity complex with the guanine exchange factor, eIF2B. This inhibits the exchange of GDP with GTP and "stalls" the preinitiation complexes on mRNAs (Sudhakar et al., 2000). Phosphorylation of as little as 20% of eIF2a significantly reduces the synthesis of most cellular proteins (Sudhakar et al., 2000). In virus-infected cells, PKR is activated by viral dsRNA. However, PKR can also be activated by Type I or II IFN by a mechanism mediated by the activated JAKs of the IFN receptor complex (Su et al., 2007), by heparin oligosaccharides, or by IL-3 withdrawal (Toth et al., 2006). PKR activation by peroxide or arsenite treatment is mediated through interaction of the activation domain of PACT with the N-terminal domain of PKR (Ito et al., 1999; Patel et al., 2000).

WNV, a member of the genus Flavivirus within the family *Flaviviridae*, was first isolated in 1937 from a febrile woman in the West Nile region of Uganda (Brinton, 2002). Until 1999, WNV was mainly confined to Southern Europe, the Middle East, Africa, West and Central Asia, Indonesia and Australia. In 1999, WNV extended into the Western hemisphere where it has since spread rapidly. The majority of WNV infections in humans are asymptomatic. Flu-like symptoms are observed in ~20% and meningitis, encephalitis and/or paralysis occur

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in less than 1% of infected individuals (Brinton, 2002; Gubler et al., 2007). The WNV genome is a positive-sense, single-stranded RNA of ~11 kb with a 5′ cap but no 3′ polyA tract. It encodes a single polyprotein that is co- and post-translationally cleaved to generate 3 structural proteins (E, prM and C) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). The steps of the viral life cycle take place in the cytoplasm. WNV infection does not lead to shut-off of cellular protein synthesis. Viral RNA replication occurs in vesicles formed by invaginations of the ER membranes (Lindenbach et al., 2007). Nascent virions are assembled through the interaction of viral structural proteins associated with ER membranes with a newly synthesized viral RNA genome followed by budding into the lumen of the ER. Virions are transported through the Golgi system to the cell surface (Brinton, 2002; Gubler et al., 2007).

PKR has been reported to play a role in NF-κB signaling and the control of cell growth through induction of p53 (Garcia et al., 2006) and also to be involved in IFN, PDGF, TNF-α, p38, JNK, STAT1 and IL-1 signaling (Garcia et al., 2006). The involvement of PKR in these multiple cellular processes requires its phosphorylation (Garcia et al., 2006). A number of cellular inhibitors have been identified that can form stable heterocomplexes with PKR and interfere with a step of the PKR activation process: (1) dsRNA recognition (C114 and RPL18), (2) dimerization ($p58^{ipk}$) or (3) autophosphorylation (Hsp70 and Hsp90) (Garcia et al., 2007). The catalytic subunit of protein phosphatase 1 alpha (PP1a) dephosphorylates PKR resulting in dimer disruption (Tan et al., 2002).

The importance of PKR as a sentinel for the antiviral innate immune response is highlighted by the many reports indicating that most known viruses have evolved mechanisms for inhibiting PKR activity (Garcia et al., 2007). Viral components can either directly inhibit PKR activation or recruit cellular PKR inhibitors. Viral proteins, including Kaposi-sarcoma herpesvirus vIRF2 and LANA2, herpes simplex virus 1 (HSV-1) Us11, Epstein-Barr virus SM, vaccinia virus E3L and hepatitis C virus NS5A and E2 proteins, directly interact with PKR and inhibit either its binding to viral dsRNA or its activation. Overexpression of human papillomavirus E6 protein was reported to induce PKR localization to P-bodies where it is sequestered (Hebner et al., 2006). Viruses, such as adenoviruses and Epstein Barr virus produce small RNA inhibitors of PKR (Langland et al., 2006; Sharp et al., 1993). However, a recent report suggests that adenovirus also overcomes PKR activation by an alternative viral protein-mediated mechanism (Spurgeon and Ornelles, 2009). Indirect mechanisms include recruitment of cellular $p58^{ipk}$ by the influenza NS1 protein into a complex with PKR where it binds to the PKR dimerization interface preventing activation (Lee et al., 1990) and recruitment of PP1a by the HSV protein $\gamma_1$34.5 to dephosphorylate eIF2a (He et al., 1998).

Consistent with our previous data showing that WNV Eg101 infection does not induce significant eIF2a phosphorylation in BHK cells (Emara and Brinton, 2007), PKR phosphorylation was not significantly induced in rodent cells after infection with either WNV Eg101 or other "natural" lineage 1 or 2 WNV strains. The activation of PKR in cells infected with many other types of viruses resulted in the evolution of viral-mediated processes to suppress PKR activation or activity. Evidence for a WNV-mediated mechanism of PKR suppression was not found. Instead, the results indicate that even though some WNV dsRNAs can activate PKR *in vitro*, WNV has developed a means to hide its dsRNA from PKR both at early and late times of the infection cycle so that PKR is not activated in infected cells.

Results

PKR phosphorylation is not induced by infection of rodent cells with natural lineage 1 or 2 strains of WNV

PKR can be activated by viral dsRNA and phosphorylates eIF2a leading to attenuation of cell translation (Garcia et al., 2007). We previously reported that WNV Eg101 infection of BHK cells did not induce significant eIF2a phosphorylation (Emara and Brinton, 2007). To determine whether the low level of eIF2a phosphorylation observed was due to a lack of PKR activation, PKR phosphorylation was initially assessed in mock-infected or WNV Eg101-infected (MOI of 5) C3H/He mouse embryo fibroblasts (MEFs) (IFN-responsive). In mock-infected MEFs treated with 100 IU/ml of Type I IFN for 24 h, the levels of PKR and Thr451 phosphorylated (P)-PKR increased significantly (Fig. 1A). Both the PKR and P-PKR levels also increased with time after infection in WNV Eg101-infected MEFs but to lower levels than with IFN treatment.

PKR protein expression is known to be upregulated in response to Type I IFN signaling (Tanaka and Samuel, 1994; Toth et al., 2006) and PKR phosphorylation can be induced through direct interactions between PKR and activated JAK1 and/or Tyk2, two components of the Type I IFN receptor complex (Su et al., 2007). We previously reported that IFN-beta expression is upregulated in WNV-infected MEFs by 12 h after infection and that 100 to 600 IU/ml of IFN beta protein are secreted into the infected cell culture fluid (Pulit-Penaloza, Scherbik and Brinton, unpublished data). To determine whether the increases in the PKR and P-PKR levels observed in C3H/He MEFs were due to IFN-mediated PKR activation, the upregulation of PKR expression and phosphorylation was compared in IFNR1−/− and control wild type 129 (129wt) MEFs infected with WNV Eg101 at a MOI of 5. Cells treated with 100 IU/ml of Type I universal IFN (PBL Biomedical laboratories, NJ) for 24 h served as a positive control. As in C3H/He MEFs, a slight increase in P-PKR and a significant increase in PKR levels compared to mock-infected cells were observed in WNV-infected and IFN-treated 129wt MEFs (Fig. 1B). In contrast, little if any increase in either PKR or P-PKR levels was observed in WNV-infected or IFN-treated IFNR1−/− MEFs (Fig. 1B). These results suggested that the small increase in P-PKR levels observed in WNV-infected MEFs was due to Type I IFN produced and secreted in response to the infection.

In contrast to the low levels of P-PKR induced by a WNV Eg101 infection in MEFs, a lineage 2/1 chimeric infectious clone-derived WNV (W956) induced much higher levels of both P-PKR and PKR (Fig. 1C). The levels of phosphorylated eIF2a were also significantly higher in cells infected with the WNV W956 virus. To determine whether the Eg101 strain was unique in its inability to induce PKR activation, IFN-non-responsive BHK cells were infected with Eg101 or another natural WNV lineage 1 (NY99 or Tx113) or lineage 2 (Mg78 or SPU) strain at a MOI of 5 for 24 h. Mock-infected BHK cells transfected with 50 μg/ml of poly(I:C) for 2 h were used as a positive control. Little if any increase in PKR phosphorylation compared to mock levels was observed in BHK cells infected with WNV Eg101 or any of the four additional WNV strains tested (Fig. 1D). Unexpectedly, a dramatic increase in PKR levels was observed in WNV Eg101-infected BHK cells but no increase in PKR levels was observed in cells infected with the other WNV viruses (Fig. 1D). The high degree of upregulation of PKR levels by a WNV Eg101 infection appears to be restricted to BHK cells since Eg101 infection of neither C3H/He nor 129wt MEFs induced greater PKR upregulation than IFN treatment and no increase in total PKR was seen in the WNV Eg101-infected IFNR1−/− MEFs. This effect also did not correlate with the lineage or virulence of the virus strains tested. Due to the Type I IFN insensitivity of BHK cells, the observed PKR upregulation by WNV Eg101 in these cells is expected to be Type I IFN-independent. Although the mechanism of PKR expression upregulation in WNV Eg101-infected BHK cells was not investigated, it was previously reported that the Sp1 and Sp3 transcription factors can upregulate PKR expression in the absence of IFN (Toth et al., 2006). Overall, the data indicate that infection of rodent cells with natural strains of WNV does not result in significant upregulation of PKR phosphorylation.

PKR localization in WNV-infected cells

PKR is typically activated in virus-infected cells by dsRNA viral replication intermediates or hairpin structures within single-stranded