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PKR activation enhances replication of classical swine fever virus in PK-15 cells

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ABSTRACT

Classical swine fever (CSF) is a highly contagious swine disease that is responsible for economic losses worldwide. Protein kinase R (PK)R is an important protein in the host viral response; however, the role of PKR in CSFV infection remains unknown. This issue was addressed in the present study using the PK-15 swine kidney cell line. We found that CSFV infection increased the phosphorylation of eukaryotic translation initiation factor (eIF)2α and its kinase PKR. However, the expression of viral proteins continued to increase. Furthermore, PKR overexpression enhanced CSFV replication, while PKR inhibition resulted in reduced CSFV replication and an increase in interferon (IFN) induction. In addition, PKR was responsible for eIF2α phosphorylation in CSFV-infected cells. These results suggest that the activation of PKR during CSFV infection is beneficial to the virus. The virus is able to commandeer the host cell's translation machinery for viral protein synthesis while evading innate immune defenses.

1. Introduction

Classical swine fever (CSF), a swine disease classified as highly contagious by the World Organization for Animal Health, is characterized by high fever, multiple hemorrhage, leukopenia, neurological dysfunction, abortion, and high mortality (Moennig et al., 2013), and is the basis for considerable economic losses worldwide. The causative agent, CSF virus (CSFV), is an enveloped virus with a single-stranded positive-sense genomic RNA that is classified as a member of the Pestivirus genus within the Flaviviridae family (Becher et al., 2003). The CSFV genome contains 5′ and 3′ untranslated region (UTRs) and a single large open reading frame encoding four structural and eight nonstructural (NS) proteins (Sheng et al., 2012). An internal ribosome entry site (IRES) located in the 5′ UTR regulates the translation of the viral genome (Fletcher and Jackson, 2002).

Translation is a key step for gene regulation in eukaryotic cells, especially when cells are stressed, such as during viral infection. Viral detection by cell sensors initiates a cascade of events that induces the transcription of IFN and other cytokines, shuts down protein synthesis and induces cell death in the cell's first line of defense to limit viral replication. PKR protein is IFN-induced gene that is present in all vertebrates. It is activated by double-stranded RNA (dsRNA) which is often a by-product of virus replication. Recent studies have found that CSFV has evolved mechanisms to inhibit IFN production in infected cells, and preventing dsRNA-mediated apoptosis, which gives rise to long-term infections (Bauhofer et al., 2007; Ruggli et al., 2005, 2009; Seago et al., 2007, 2010); however, the role of PKR in CSFV infection has not been elucidated.

Eukaryotic translation initiation factor (eIF)2 is a key regulator of translation initiation. PKR is one of the four eIF2α kinases – the others being general control non-derepressible 2, double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase, and heme-regulated inhibitor kinase – that phosphorylate eIF2α in response to viral dsRNA, heme levels, misfolded proteins, and amino acid deprivation, respectively (Taylor et al., 2005). PKR binds to dsRNA and undergoes auto-phosphorylation, which activates the kinase that phosphorylates eIF2α at Ser51, thereby inhibiting translation initiation and protein synthesis in various types of viral infection (Cole, 2007; Garcia et al., 2006). This constitutes the basic mechanism by which PKR exerts its antiviral activity on a wide spectrum of DNA and RNA viruses.

Some viruses can evade the antiviral function of PKR. Rotavirus infection can induce PKR activation, eIF2α phosphorylation, and the modification of the cellular translation machinery while circumventing the host immune response. The phosphorylation of eIF2a has been shown to block translation initiation of most

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cellular proteins without affecting viral replication (Lopez and Arias, 2012; Rojas et al., 2010). In hepatitis C virus (HCV)-infected cells, PKR activation may be advantageous to the virus owing to reduced IFN production and consequent suppression of major histocompatibility complex I expression. In addition, PKR is responsible for the resistance of HCV to IFN treatment (Arnaud et al., 2010, 2011; Garaigorta and Chisari, 2009; Kang et al., 2014). In the Flaviviridae family, activated PKR was found to inhibit infection by West Nile virus (WNV) but not by dengue virus (Jiang et al., 2010).

Recent reports have shown that upon phosphorylation, eIF2α can still associate with the IIId domain of the CSFV IRES, which delivers Met-tRNA to the P site of a 40S ribosomal subunit to form the 48S initiation complex (Friis et al., 2012; Hashem et al., 2013; Jackson et al., 2010; Locker et al., 2007; Pestova et al., 2008). Despite many studies, the function of PKR in CSFV infection is still unknown. In the present study, we investigated whether CSFV infection can activate PKR and eIF2α, and examined the role of PKR in this process. The effect of PKR on CSFV replication and IFN induction was assessed by over-expressing or inhibiting PKR. Our results show that CSFV infection leads to activation of PKR and eIF2α and enhances viral replication.

2. Materials and methods

2.1. Reagents

Primary antibodies against the following proteins were used in this study: PKR (BS3653), phospho-PKR^{T446} (BS4789), and myxovirus resistance (MX)1 (BS6674) (all from BioWorld, Visalia, CA, USA); eIF2α (SC-11386) and phospho-eIF2α^{S51} (SC-12412) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA); CSFV E2 (WH303; Median Diagnostics, Chuncheon, Korea); β-actin (Beyotime, Beijing, China, AA128); and green fluorescent protein (GFP) (1533-1; Epitomics, Burlingame, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit (BS13278) and anti-mouse (BS12478) secondary antibodies were from BioWorld. Alexa Fluor 555-labeled donkey anti-rabbit (A0453) and Alexa Fluor 488-labeled goat anti-mouse (A0428) secondary antibodies were from Beyotime. 2-Aminopurine (2-AP; A2380) and poly(I:C) (P1530) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The pEGFP-PKR and pEGFP-N1 vectors were prepared in our laboratory (Tian and Mathews, 2001). Monoclonal antibodies against the NS5A, NS3, and N^{pro} proteins of CSFV were provided by Dr. Xinglong Yu (Veterinary Department, Hunan Agricultural University, China). PKR and scrambled short hairpin (sh)RNA were designed by Cyagen Biosciences Inc. (Santa Clara, CA, USA). The PKR shRNA sequence 5′-GCA GAA CTT CTT CAC ATA TGT-3′ was inserted into the PLenti X1-puro-shRNA-eGFP vector.

2.2. Cells and virus

The swine kidney cell line PK-15 (CCL-33; American Type Culture Collection, Manassas, VA, USA) was maintained in complete Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Cells were incubated at 37 °C with 5% CO_2. The CSFV Shimen strain used in this study was isolated from swine exhibiting typical CSF symptoms and was propagated in two cell cultures in medium containing 2% FBS, and then stored in our laboratory. Virus titers were determined using a monoclonal antibody against CSFV E2 as previously described (Pei et al., 2014). The multiplicity of infection (MOI) was calculated based on the virus titer. CSFV was inactivated by irradiating cells with ultraviolet (UV) light for 30 min at room temperature. The infectivity of UV-treated CSFV was confirmed by reverse transcription (RT)-PCR to detect propagated virus, and the MOI was found to be the same as that of uninfected cells.

2.3. Viral infection

PK-15 cells were grown to ~80% confluence in cell culture plates and infected with CSFV at various MOIs. The mock-infected control was treated with phosphate-buffered saline (PBS). After 1 h, the inoculum was removed by aspiration. Cells were then washed twice with PBS and incubated in complete medium at 37 °C for various times until harvesting.

2.4. Western blotting

Cell monolayers were washed twice in PBS and incubated on ice with radioimmunoprecipitation lysis buffer (P0013B; Beyotime) containing 1 mM phenylmethylsulfonyl fluoride (ST506; Beyotime) for 10 min. Lysates were then centrifuged at 12,000 rpm for 20 min at 4 °C, and protein concentration was determined using a bicinchoninic acid assay kit (23227; Thermo Scientific, Waltham, MA, USA). Equal amounts of protein sample were boiled for 5 min in 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins (20 μg) were separated by 12% SDS-PAGE, electrotransferred to polyvinylidene fluoride membranes (IPFL00010; Merck Millipore, Billerica, MA, USA), and then blocked in 5% non-fat milk for 2 h at room temperature. Membranes were then incubated with primary antibodies at 4 °C overnight, washed three times for 10 min with PBS containing 0.5% Tween-20, and incubated with secondary antibody at 37 °C for 1 h. Protein bands were detected by enhanced chemiluminescence (P0018; Beyotime) and imaged using a CanoScan LiDE 100 scanner (Canon, Tokyo, Japan); band intensity was quantitated using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Confocal immunofluorescence microscopy

PK-15 cells were seeded on EZ SLIDEs (Merck Millipore, PEZGS0816) and infected with CSFV at MOI of 1 or treated with PBS (mock-infected control). Cells were transfected with Lipofectamine 2000 (11668027; Life Technologies, Carlsbad, CA, USA) and 0.8 μg poly(I:C) as negative and positive controls, respectively. After 24 h of incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cell monolayers were permeabilized with 0.1% Triton X-100 for 30 min. Cells were then blocked with PBS containing 5% bovine serum albumin for 30 min at room temperature, and incubated for 1 h with rabbit polyclonal anti-eIF2α (1:50) and mouse monoclonal anti-NS5A (1:100) antibodies in PBS at 37 °C, followed by a 1-h incubation in PBS containing Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit secondary antibodies (1:200). Fluorescence was visualized using an LSM 780 confocal microscope (Zeiss, Jena, Germany).

The translocation of eIF2α was determined as a ratio of nuclear to cytoplasmic localization of the protein. Quantification was performed using ImageJ software (National Institutes of Health; Bethesda, MD, USA). Images were acquired of three random fields from each sample. The cytoplasm and nuclear regions were determined by using ImageJ binary mask and image subtraction calculation. All samples in the same experiment were recorded with the same microscopic settings so that the images were comparable. Duplicate slides were made of each sample and three independent experiments were conducted (Noursadeghi et al., 2008; Zhu and Carver, 2012).

To assess the effects of PKR activation in CSFV-infected cells, PK-15 cells grown to ~70% confluence on EZ SLIDEs were transfected with pEGFP-PKR plasmid using Lipofectamine 2000 reagent