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Degradation of CREB-binding protein and modulation of type I interferon induction by the zinc finger motif of the porcine reproductive and respiratory syndrome virus nsp1α subunit

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ABSTRACT

Non-structural protein (nsp) 1 of PRRS virus is a viral antagonist for type I interferons (IFNs), and in cells expressing nsp1, CREB-binding protein (CBP) is degraded. nsp1 is auto-processed into nsp1α and nsp1β subunits and in the present study we show that the nsp1α subunit was responsible for CBP degradation. The nsp1α subunit contains three distinct functional motifs; a papain-like cysteine protease α (PCPα) motif, an N-terminal zinc finger motif (ZF1), and a newly reported C-terminal zinc finger motif (ZF2). To study the structure function of nsp1α and its IFN antagonism, these motifs were individually mutated and the mutants were examined for their IFN suppression ability. The mutations that destroyed the PCPα activities (C76S, H146Y, and C76S/H146Y) did not affect the IFN suppressive activity of nsp1α, indicating that the cysteine protease activity did not participate in IFN suppression. The mutations of C70S, C76S, H146Y, and/or M180I which coordinated the ZF2 motif also did not alter IFN suppression. However, the mutations of C8S, C10S, C25S, and/or C28S for the ZF1 motif impaired the IFN antagonism of nsp1α, demonstrating that ZF1 was the essential element of nsp1α for IFN suppression. Wild-type nsp1α localized in the both nucleus and cytoplasm, but the ZF1 mutants that lost the IFN suppressive activity did not localize in the nucleus and remained in the cytoplasm. Consistent with their cytoplasmic distribution, CBP was not degraded by these mutants. Our results indicate that the ZF1 motif of nsp1α plays an important role for IFN regulation and further demonstrate that the CBP degradation is likely the key mechanism for IFN suppression mediated by the nsp1α subunit protein of PRRS virus.

1. Introduction

The type I interferon (IFN) system is considered a key component of the innate immune response and represents one of the first lines of defense against virus infection (Samuel, 2001). The IFN-induced signal transduction leads to the expression of more than 300 IFN-stimulated genes (ISG) that contribute to the antiviral state of host cells, directly or indirectly (Sadler and Williams, 2008). Type I IFNs are induced directly in response to virus infection and viral components are recognized by two distinct pattern recognition receptors (PRRs); toll-like receptors (TLRs) and RIG-like receptors (RLRs) (reviewed in Yoo et al., 2010). After the binding of pathogen-associated molecular patterns (PAMPs) to PRRs, ensuing signal transduction leads to the activation of IFN regulatory factor 3 (IRF3) and the release of nuclear factor-kappa B (NF-κB) from its inhibitor IκB (Baccala et al., 2007). IRF3 is activated through phosphorylation by IKKε and/or TBK1 kinases, and the phosphorylation results in the IRF3 dimerization and conformational switch, exposing nuclear localization signal (NLS) for subsequent nuclear translocation. By phosphorylation, an auto-inhibitory (feature of IRF3 that prevents its interaction with CREB (cyclic AMP responsive element binding)-binding protein (CBP) is removed) (Panne et al., 2007; Takahasi et al., 2010). For NF-κB-mediated signaling, it is normally held in the cytoplasm by association with the inhibitor of NF-κB (IκB). Once the signaling cascade contributes to the phosphorylation of IκB and its subsequent ubiquitination and degradation by proteasomes, NF-κB is detached from IκB and translocates to the nucleus (Murray, 2007). After nuclear translocation, activated IRF3 and NF-κB exert modulation on the activation of IFN-β promoter. Within the IFN-β promoter, four regulatory *cis* elements are found, namely, the positive regulatory domains (PRDs) I, II, III, and IV. PRD I/III, PRD II and PRD IV are binding sites for IRFs, NF-κB, and ATF-2/c-Jun (AP-1), respectively (Honda et al., 2006). The expression of IFN-β requires the assembly of these regulatory

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factors on PRDs to form an enhanceosome complex, which recruits CBP to instigate IFN production (Randall and Goodbourn, 2008).

Porcine reproductive and respiratory syndrome (PRRS) is an emerged and re-emerging swine disease featured by severe reproductive losses, post-weaning pneumonia and increased mortality. PRRS was first reported in the Unites States in 1987 and subsequently in Europe in 1990, and has since become the most economically significant disease to pig production worldwide (Chand et al., 2012). PRRS virus (PRRSV) is the etiological agent placed in the family Arteriviridae in the order Nidovirales, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). PRRSV possesses a single-stranded positive-sense RNA genome of 15 kb in length, harboring 10 ORFs: ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7 (Meulenberg et al., 1993; Wootton et al., 2000), plus a newly identified ORF5a (Firth et al., 2011; Johnson et al., 2011). Mediated by the ribosomal frame-shifting signal in the ORF1a/ORF1b overlapping region, ORF1a and ORF1b synthesize two polyproteins, pp1a and pp1ab, which are proteolytically cleaved into 14 non-structural proteins (nsp) by papain-like cysteine protease (PCP) α and PCPβ in nsp1, poliovirus 3C-like cysteine protease (CP) in nsp2, and serine protease (SP) in nsp4 (reviewed by Fang and Snijder, 2010). The remaining ORFs code for eight different structural proteins: glycoprotein (GP) 2, small envelopes (E), GP3, GP4, GP5, ORF5a, membrane (M), and nucleocapsid (N) proteins (Firth et al., 2011; Johnson et al., 2011; Meulenberg et al., 1993; Wootton et al., 2000).

PRRSV seems to suppress the production of type I IFNs in infected pigs (Albina et al., 1998). IFNs are negligible in the lungs of pigs where PRRSV actively replicate in vivo, and the induction of IFN is poor in virus-infected PAMs (porcine alveolar macrophages) and MARC-145 cells in vitro. PRRSV can establish a persistent infection for up to 6 months. It seems that PRRSV has evolved to modulate the host antiviral response via several strategies such as delayed generation of neutralizing antibody and suppressed IFN production (Allende et al., 2000; Diaz et al., 2005; Lee et al., 2004; Luo et al., 2008). The molecular basis for modulation of type I IFN production by PRRSV has recently been studied and five viral proteins have been identified to date as IFN antagonists: N as a structural protein and four non-structural proteins, nsp1α, nsp1β, nsp2 and nsp11 (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Sagong and Lee, 2011; Sun et al., 2010; Yoo et al., 2010).

Among these, nsp1α is the first viral protein synthesized during virus-infection. nsp1α is a multifunctional regulatory protein, containing an N-terminal zinc finger (ZF) motif, designated as ZF1 in the present study, and the papain-like cysteine protease (PCP) α domain. The ZF1 motif of nsp1α belongs to the 4-Cys (4-C) ZF superfamily, and mutations in the ZF1 motif selectively eliminate subgenomic (sg) mRNA synthesis, while genomic RNA synthesis is either not affected or even increased (Sun et al., 2009; Tijms et al., 2007). The PCPα domain conducts self-processing of nsp1, yielding nsp1α and nsp1β subunits. This auto-cleavage is essential for synthesis of PRRSV sg mRNA but does not affect viral genome replication (Kroese et al., 2008). The crystallographic study for nsp1α has identified a second zinc finger motif in the C-terminal region, designated ZF2 in the present study, and that the active site residues for PCPα participate in the ZF2 configuration to coordinate a zinc atom (Sun et al., 2009). The biological function of ZF2 is unknown.

Recent studies have shown that PRRSV nsp1 suppresses the production of type I IFNs in PRRSV-infected cells and gene-transfected cells, and its cleavage products, nsp1α and nsp1β, are capable of blocking the induction of type I interferon by targeting both IRF3- and NF-κB-mediated production pathways (Beura et al., 2010; Chen et al., 2010; Song et al., 2010). Further investigations showed CBP degradation in the presence of nsp1, and the degradation was proteasome-dependent (Kim et al., 2010). In the present study, we investigated the basis for the type I IFN suppression mediated by nsp1 and showed that nsp1α subunit alone was responsible and sufficient for CBP degradation. We also determined the importance of ZF1 for IFN suppression and provided evidence that the CBP degradation was likely the basis for IFN suppression mediated by nsp1α of PRRSV.

2. Materials and methods

2.1. Cells and viruses

HeLa cells (NIH AIDS Research and Reference Reagent Program, Germantown, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc., Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) in a humidified incubator with 5% CO_2 at 37 °C. The PA8 strain of the North American genotype PRRSV (Wootton et al., 2000) was used throughout the study. The full-length genomic sequence of PA8 shares 99.2% identity with the prototype PRRSV VR2332 of the North American genotype (Nelsen et al., 1999).

2.2. Plasmids and DNA cloning

The plasmids pFLAG-nsp1 and pFLAG-nsp1α contain the full-length nsp1 and nsp1α genes, respectively, fused with an N-terminal FLAG tag (Song et al., 2010). The full sequence of nsp1β including the N-terminal FLAG tag was amplified by PCR using pFALG-nsp1 as a template using the upstream primer (5′-CCGAATTCACCATG*GATTACAAGGATGACGACGATAAG*GCTACTGTCTATGACATTGGTCATGGCTAC-3′, where the EcoRI recognition sequence is underlined and the FLAG tag is italicized and underlined) and the downstream primer (5′-GGCTCGAGCTAGCCGTACCACTTGT-3′, where the XhoI sequence is underlined). The PCR fragment was cloned into pXJ41 mammalian expression vector after digestion with XhoI and EcoRI (Xiao et al., 1991). Mutant plasmids were generated using PCR-based site-directed mutagenesis as described below using plasmid pFLAG-nsp1α as a template and a set of primers listed in Table 1. pIFN-β-Luc, p4xIRF3-Luc and pTATA-Luc, were kindly provided by Stephan Ludwig (Ehrhardt et al., 2004; Institute of Molecular Medicine, Heinrich Heine Universtät, Düsseldorf, Germany). The pIFN-β-luc plasmid contains the luciferase reporter gene placed under the IFN-β promoter. The p4xIRF3-Luc construct contains four copies of the IRF3 specific PRDI/III domain of the IFN-β promoter in front of the luciferase reporter gene. The plasmid pPRDII-Luc contains 2 copies of the NF-κB binding region PRD II of the IFN-β promoter in front of the luciferase gene and was kindly provided by Stanley Perlman (University of Iowa, IA; Zhou and Perlman, 2007). The plasmid pISRE-Luc contains the IFN stimulated response element (ISRE) binding sequence upstream of the luciferase reporter gene (Stratagene, La Jolla, CA, USA). The pRL-TK plasmid (Promega) contains the renilla luciferase reporter under control of the herpes simplex virus thymidine kinase (HSV-tk) promoter and was included in all experiments to serve as an internal control.

2.3. Antibodies and chemicals

Polyinosinic:polycytidylic [poly (I:C)] and anti-Flag MAb (M2) were purchased from Sigma (St. Louis, MO, USA). Anti-β-actin MAb (sc-47778), anti-CBP MAbs (sc-7300), and anti-PML PAb (sc-5621) were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Anti-ISG15 PAb was purchased from Thermo Scientific Pierce (Rockford, IL, USA). The peroxidase-conjugated Affinipure goat anti-mouse IgG and peroxidase-conjugated Affinipure goat anti-rabbit IgG were purchased from Jackson Immuno Research