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# Nuclear export signal of PRRSV NSP1α is necessary for type I IFN inhibition

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ABSTRACT

The nonstructural protein 1α (NSP1α) of porcine reproductive and respiratory syndrome virus (PRRSV) is a nucleo-cytoplasmic protein that suppresses the production of type I interferon (IFN). In this study, we investigated the relationship between the subcellular distribution of NSP1α and its inhibition of type I IFN. NSP1α was found to contain the classical nuclear export signal (NES) and NSP1α nuclear export was CRM-1-mediated. NSP1α was shuttling between the nucleus and cytoplasm. We also showed that the nuclear export of NSP1α was necessary for its ability for type I IFN inhibition. NSP1α was also found to interact with CBP, which implies a possible mechanism of CBP degradation by NSP1α. Taken together, our results describe a novel mechanism of PRRSV NSP1α for type I IFN inhibition and suppression of the host innate antiviral response.



## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerging and re-emerging swine disease that severely affects swine populations worldwide. The etiological agent is the PRRS virus (PRRSV), a member of the family *Arteriviridae* in the order *Nidovirales* (http://www.ictvonline.org/virusTaxonomy.asp). PRRSV possesses a single-stranded and positive-sense RNA genome of 15 kb in length, which includes at least 10 ORFs; namely, ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3–7, and ORF5a (Dokland, 2010; Fang and Snijder, 2010; Johnson et al., 2011; Ozato et al., 2008). ORF1a and ORF1b are comprised of 75% of the total genome and code for two polyproteins, pp1a and pp1b, which are proteolytically cleaved into 14 non-structural proteins (NSPs) by papain-like cysteine protease (PLP) α in NSP1α, PLPβ in NSP1β, poliovirus 3C-like cysteine protease (PLP2) in NSP2, and serine protease (SP) in NSP4. The remaining ORFs code for 8 structural proteins, namely, glycoprotein 2 (GP2), small envelope protein (E), GP3, GP4, GP5, GP5a, membrane (M) and nucleocapsid (N) proteins (Dokland, 2010; Johnson et al., 2011; Meulenberg, 2000; Ozato et al., 2008).

Virus infection of host cells leads to initiation of antiviral innate immune responses, which lead to expression of type I IFNs and pro-inflammatory cytokines. Type I IFNs (IFN-α/β) are considered as key components of the innate immunity and provide the first barrier of defense against virus infections (Gale and Sen, 2009). During infection, viral pathogen-associated molecular patterns (PAMPs), mostly viral nucleic acids, are initially recognized by host pattern-recognition receptors (PRRs); subsequently, signaling pathways are activated, which lead to transcription of IFNs (Luo et al., 2008; Patel et al., 2010). Viruses have evolved strategies to inhibit aspects of PRRs-mediated signaling to evade the host antiviral mechanism. PRRSV is a weak inducer of IFNs *in vitro* and *in vivo*. IFNs are poorly expressed in PRRSV-infected MARC-145 cells and porcine alveolar macrophages (PAMs), which are the primary target cells of the virus (Miller et al., 2004; Sagong and Lee, 2011). Moreover, IFNs are negligible in the lungs of pigs, where PPRSV actively replicates *in vivo* (Gomez-Laguna et al., 2010). The molecular mechanism of PRRSV to inhibit type I IFN production has been studied; at least six viral proteins (NSP1α,

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NSP1β, NSP2, NSP4, NSP11, and N protein) were identified to possess an inhibitory effect on type I IFN production (Beura et al., 2010; Chen et al., 2014; Sagong and Lee, 2011).

As the first protein synthesized during infection, NSP1α is generated through self-cleavage at Met180-Ala181 and plays important roles throughout the course of PRRSV replication. In addition to its function as an indispensable factor for genome replication and protease in replication, NSP1α functions as an IFN antagonist; which blocks NF-κB activation via suppression of IκB phosphorylation (Song et al., 2010) and inhibits type I interferon transcription by degrading CREB-binding protein (CBP) (Han et al., 2013, 2014). Further analysis showed the nuclear localization of NSP1α was related to its IFN-suppressive activity (Han et al., 2014).

In this study, the nucleo-cytoplasmic shuttling scheme of NSP1α and its relationship to IFN inhibition were determined. The results revealed the shuttling scheme of NSP1α and the importance of the nuclear export of NSP1α for inhibition of its type I IFN.

## 2. Materials and methods

### 2.1. Cells, viruses, and chemicals

MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml in a humidified incubator with 5% $CO_2$ at 37 °C. The PRRSV SD-JN strain was used to clone NSP1α (Wu et al., 2009). MG132, leptomycin B (LMB), and cyclohexamide (CHX) were purchased from Beyotime (Jiangsu, China).

### 2.2. Plasmids

The pGL3-promoter and pRL-TK were purchased from Promega (Madison, WI, USA). The pIFN-β-luc plasmid was constructed by cloning −281 to +19 region of the IFN-β promoter into the pGL3-promoter vector as described previously (Beura et al., 2010; Chen et al., 2014). The pEGFP-N1 and pCMV-HA were purchased from Clontech (Palo Alto, CA, USA). The NSP1α gene of the PRRSV SD-JN strain (Wu et al., 2009) was amplified by RT-PCR and cloned in the mammalian expression vector pEGFP-N1 (Clontech) downstream of the green fluorescent protein (GFP) and named NSP1α-GFP; or cloned in the pCMV-HA vector (Clontech) fused with the N-terminal HA-tag, and named HA-NSP1α (Table 1). Plasmids with NES mutation or deletion from NSP1α-GFP or HA-NSP1α were constructed by replacing eight leucine residues in NES (LSARSLLPLNLQVPELGVLGL) with alanine or deleting NES using the Fast Mutagenesis system according to the manufacturer's instructions (Trans, China). Primers used in cloning and mutations are listed in Table 1. The nuclear localization sequence (PKKKRKV) of SV40 large T antigen (Kalderon et al., 1984) was cloned into the mammalian expression vector pEGFP-N1 to obtain plasmid NLS-GFP. The NES sequence of the NSP1α or MEK1 protein (Jaaro et al., 1997) was then inserted into the NLS-GFP plasmid (Figs. 1C and 2A). All constructs were sequenced to confirm the correct in-frame insertion of individual genes.

### 2.3. Transfection and luciferase reporter assay

MARC-145 cells were seeded in 12-well plates and grown to 70–80% confluency. The cells were transfected with 250 ng of the pIFN-β-luc luciferase reporter plasmid, 2.5 ng of pRL-TK, and 250 ng of NSP1α-GFP by using Lipofectamine™ 3000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). An empty vector was added to the transfection mixture to keep the total DNA amount constant. At 24 h post-transfection, the cells were transfected with 1 μg poly (I:C) (Sigma-Aldrich, St. Louis, MO, USA). After 12 h, luciferase assay was performed using a dual luciferase assay system (Promega). Inhibition of transcriptional efficiency was evaluated as relative luciferase activity, which was calculated by normalizing Firefly luciferase to *Renilla* luciferase activities according to the manufacturer's instructions. All assays were repeated at least three times, with each experiment performed in triple.

### 2.4. Co-immunoprecipitation (Co-IP) assay

To determine the interaction between CRM-1 (karyopherin chromosome region maintenance-1) and NSP1α, MARC-145 cells grown in a 10-cm dish were transfected with 5 μg HA-NSP1α for 2 days. After LMB treatment for 10 h, the cells were washed twice with PBS and collected. The cells were then resuspended in lysis buffer [50 mM Tris (pH 7.4) containing 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate and complete protease inhibitor (Roche, Rotkreuz, Switzerland)]. The cell lysates were clarified by centrifugation at 12,000g for 10 min at 4 °C. The supernatant was subjected to Co-IP experiments using HA-Tag IP/Co-IP kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After two washes with TBST [25 mM Tris (pH7.2) containing 150 mM NaCl and 0.05% Tween-20], Sepharose beads were incubated with cell lysis for 2 h at 4 °C, and washed with

**Table 1**
Primers used in the study.

| Primer name | Sequence (5′ – 3′) | Purpose |
|---|---|---|
| HA-NSP1α-F | CG*GAATTC*CCATGTCTGGGATACTTGATCG (*Eco*RI) | HA-Nsp1α plamid clone |
| HA-NSP1α-R | GGA*AGATCT*TCACATAGCACACTCAAAAGGGC (*Bgl*II) | |
| NSP1α-GFP-F | TTT*GAATTC*CCATGTCTGGGATACTTGATCGGTGCACGT (*Eco*RI) | NSP1α-GFP plasmid clone |
| NSP1α-GFP-R | GGG*GGATCC*CGCATAGCACACTCAAAAGGGCAAAAGTC (*Bam*HI) | |
| MT-NSP1α-F | CCT**GC**GAAT**GC**CCAAGTTCCTGAG**GC**TGGGGTG**GC**GGGT**GC**ATTTTATAGGCCCGAAGA | Leu to Ala mutation in HA-NSP1α and NSP1α-GFP |
| MT-NSP1α-R | GGAACTTGG**GC**ATTC**GC**AGGA**GC**G**GC**AGACCGTGCACTGgcACATCGTGTGCAGTAGAC | |
| DT-NSP1α-F | TGCACACGATGTTTTTATAGGCCCGAAGAGCCA | NES of NSP1α deletion in HA-NSP1α and NSP1α-GFP |
| DT-NSP1α-R | GGGCCTATAAAAACATCGTGTGCAGTAGACCT | |
| NLS-GFP-F | ***AAAAAGAAGAGAAAGGTA***GATCTCGAGCTCAAGCTT | NLS of SV40 Large T antigen insertion |
| NLS-GFP-R | ***CTTTCTCTTCTTTTTTGG***TGAGTCCGGTAGCGCTAG | |
| NLES-F | ***GAAGCTGGAGGAGCTAGAGCTTGATGAG***CGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC | NES of MEK1 insertion to NLS-GFP |
| NLES-R | ***CTAGCTCCTCCAGCTTCTTCTGCAAGGCCAT***AAGCTTGAGCTCGAGATCTACCTTTCTCTTCTTTTTTG | |
| NES of NSP1α-F | ***CTCCAAGTTCCTGAGCTTGGGGTGCTGGGTCTA***CGAATTCTGCAGTCGACG | NES of NSP1α insertion to NLS-GFP |
| NES of NSP1α-R | ***GCTCAGGAACTTGGAGATTCAGAGGAAGGAGAGACCGTGCACTGA***GAAGCTTGAGCTCGAGATC | |

Restriction endonuclease sites were underlined in italics. The NLS of SV40 large T antigen, NES of MEK1, or NES of NSP1α were both in italics and boldface. The mutated sites were underlined in boldface.