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Cellular Hsp27 interacts with classical swine fever virus NS5A protein and negatively regulates viral replication by the NF-κB signaling pathway

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ABSTRACT

Classical swine fever virus (CSFV) nonstructural protein NS5A is a multifunctional protein functioning in regulation of viral genome replication, protein translation and assembly by interaction with viral or host proteins. Here, heat shock protein 27 (Hsp27) has been identified as a novel binding partner of NS5A by using His tag "pull down" coupled with shotgun LC-MS/MS, with interaction of both proteins further confirmed by co-immunoprecipitation and laser confocal assays. In PK-15 cells, silencing of Hsp27 expression by siRNA enhanced CSFV replication, and upregulation of Hsp27 inhibited viral proliferation. Additionally, we have shown that overexpression of Hsp27 increased NF-κB signaling induced by TNFα. Blocking NF-κB signaling in PK-15 cells overexpressing Hsp27 by ammonium pyrrolidinedithiocarbamate (PDTC) eliminated the inhibition of CSFV replication by Hsp27. These findings clearly demonstrate that the inhibition of CSFV replication by Hsp27 is mediated via the NF-κB signaling pathway.

1. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious swine disease causing significant economic losses to the pig industry worldwide. Symptoms range from typical to atypical or even clinically inapparent depending on the virulence of the virus strain. Infections with virulent strains result in acute hemorrhagic fever and immunosuppression with high morbidity and mortality (Thiel et al., 1996; Moennig and Plagemann, 1992). Low virulent strain infection causes a chronic or subclinical disease with atypical symptoms or inapparent clinical manifestations (Muñoz-González et al., 2015).

CSFV is a member of the genus *Pestivirus* within the family *Flaviviridae* (Simmonds et al., 2011). CSFV is a small enveloped virus with a single, positive-stranded RNA genome containing a 5′ untranslated region (5′UTR) harboring an internal ribosome entry site (IRES), a single large open reading frame (ORF) and a 3′ untranslated region (3′ UTR). The ORF encodes a polyprotein containing 3898 amino acids which is cleaved by cellular and viral proteases to produce 4 structural proteins (protein C and glycoproteins E^{rns}, E1, E2) and 8 nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Tautz et al., 2015).

Pestiviral nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B are essential for viral RNA replication (Behrens et al., 1998). NS5A is an essential component of the replication complex and can be complemented *in trans* (Grassmann et al., 2001). CSFV NS5A is a 55KD phosphorylated protein functioning in viral genome replication, protein translation and assembly by interaction with viral or host factors. Reports show that CSFV NS5A regulates viral RNA replication by binding to the viral genome 3′-UTR or by modulating NS5B RdRp activity (Sheng et al., 2012a; Chen et al., 2012). CSFV NS5A also regulates viral translation by repression of CSFV IRES activity in a dose-dependent manner (Xiao et al., 2009), which can be relieved by binding of NS5B protein to NS5A (Sheng et al., 2012b). NS5A also plays a critical role in the assembly and production of infectious CSFV particles by interaction with core protein (Sheng et al., 2014) and with cellular annexin A2 protein (Sheng et al., 2015). In addition, NS5A suppresses the secretion of inflammatory cytokines induced by poly(I:C) through inhibition of the NF-κB signaling pathway (Dong and Tang, 2016). Nevertheless, the role of NS5A in the viral replication cycle still require clarification.

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Heat shock proteins function as intracellular chaperones and play important roles in protein folding, assembly, proteasomal degradation, protein translocation and cell apoptosis. Heat shock protein 27 (Hsp27) is a member of a small heat shock protein family that includes aA- and aB-crystallins, all containing a conserved C-terminal domain (Kappe et al., 2003). Hsp27 is a multifunctional protein acting as both a protein chaperone and an antioxidant, involved in the inhibition of apoptosis and actin cytoskeletal remodeling (Acunzo et al., 2014). Parcellier et al. (2003) reported that Hsp27 is an ubiquitin-binding protein, mediating IκBα proteasomal degradation. It has also been reported to interact with viral proteins and to be involved in viral replication (Choi et al., 2004; Liu et al., 2014). Here we demonstrate that Hsp27 interacts with CSFV NS5A and negatively regulates viral replication through the NF-κB signaling pathway.

2. Results

2.1. Identification of cellular proteins interacting with NS5A protein

Purified His-NS5A or His-tag protein coupled to Dynabeads (Invitrogen, Carlsbad, CA, USA) were incubated with PK-15 cell lysates in a His-tag pull-down assay. Proteins specifically binding to His-NS5A or His-tag were visualized by SDS-PAGE and Coomassie brilliant blue (G250) staining (Fig. 1A). The gels in lanes containing His-NS5A or His tag protein and their binding partners of host were excised, destained and digested with trypsin, the peptide mixtures were extracted and identified by using LC-MS/MS. Among the proteins binding to His-NS5A but not to the His-tag, Hsp27 was selected for further investigation because of its involvement in the replication of other viruses (Choi et al., 2004; Liu et al., 2014).

2.2. Hsp27 interacts with NS5A

Western blotting probing for Hsp27 that copurified with fusion protein His-NS5A was performed using anti-Hsp27 antibody. Fig. 1B shows that Hsp27 associated with NS5A rather than the His-tag.

To determine the interaction using cell cultures, PK-15 cells were transfected with recombinant vectors p3xFLAG-NS5A or p3xFLAG-CMV-10. Forty-eight h post-transfection, cell extracts were immunoprecipitated with anti-FLAG antibody. Precipitated proteins were boiled in an SDS loading buffer and subjected to western blotting using anti-Hsp27 antibodies. A positive result was observed only in precipitates containing fusion protein FLAG-NS5A (Fig. 1C). These data further show the association of Hsp27 with NS5A.

2.3. Co-localization of CSFV NS5A with cellular Hsp27 in PK-15 cells

PK-15 cells were transfected with p3xFLAG-NS5A or p3xFLAG-cmv-10 vector, 48 h after transfection, subcellular localization of NS5A and Hsp27 was analyzed by immunofluorescence staining and confocal microscopy. Fig. 2 shows a clear co-localization of CSFV NS5A with cellular Hsp27 within cells expressing NS5A.

2.4. Downregulation of Hsp27 by siRNAs facilitates viral replication

PK-15 cells were transfected with siRNA targeting Hsp27 or non-targeting control siRNA (NC). Western blot analysis showed that in PK-15 cells transfected with siRNA-1, siRNA-2 or siRNA-3 either separately or together (Fig. 3A upper) Hsp27 expression was significantly decreased compared with that in untreated (UT) cells or control cells transfected with NC (Fig. 3A lower). In addition, MTT assay showed knockdown of Hsp27 does not result in a significant decrease in cell viability.

Twenty four h following transfection with pooled siRNA-1, siRNA-2 and siRNA-3, PK-15 cells were infected with virulent CSFV Shimen strain. Forty eight h later, quantitative analyses of progeny virus, viral genome and viral protein E2 were performed. Results showed increases in viral genome copies, titers of progeny virus and viral protein E2 in siRNA treated cells compared with that in NC treated cells and UT cells. (Fig. 3B, C and D).

2.5. Upregulation of Hsp27 expression inhibits CSFV proliferation

In PK-15 cells transfected with pCDNA-Hsp27 vector, overexpression of Hsp27 was observed (Fig. 4A). MTT assay showed that overexpression of Hsp27 did not cause significant damage to cell viability. Forty eight h following infection of cells overexpressing Hsp27 with CSFV Shimen, analysis of viral replication showed that progeny virus titers, E2 protein and viral genome copies were all decreased compared to that in control cells (Fig. 4B,C,D).

2.6. NS5A reduces the expression of Hsp27 protein

To investigate whether NS5A modulates Hsp27 expression, 293T cells in 24-well cell culture plates were co-transfected with p3xFLAG-NS5A and pCDNA-Hsp27 or with p3xFLAG-CMV-10 and pCDNA-Hsp27. At 48 h after transfection, Hsp27 expression was determined by western blotting. Results indicated a reduction of Hsp27 protein in cells expressing NS5A compared to that in cells without NS5A (Fig. 5).

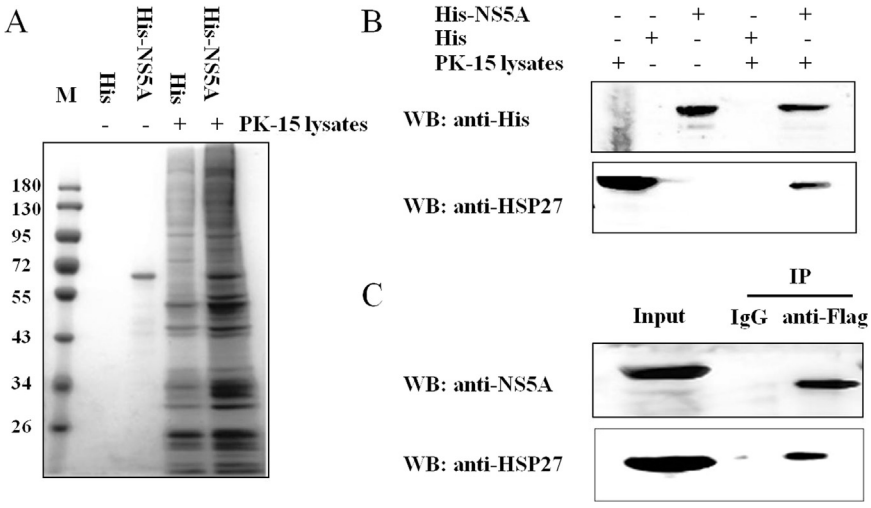

Fig. 1. Hsp27 interacts with NS5A. (A) His tag and His-NS5A proteins were purified with Dynabeads and incubated with (+) or without (−) PK-15 cell lysates for pulldown assay. Purified proteins and pulldown proteins were separated by 12% SDS-PAGE, followed by Coomassie brilliant blue staining. M: protein marker (KDa). (B) Pulled-down proteins were analyzed by western blotting with anti-His (top), anti-Hsp27 (foot) antibodies. (C) PK-15 cells were transfected with p3xFLAG-NS5A recombinant plasmids, followed by immunoprecipitation (IP) with anti-FLAG antibody or normal mouse IgG. The precipitated proteins were probed by western blotting (WB) using anti-NS5A or anti- Hsp27 antibody.