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The eukaryotic translation initiation factor 3 subunit E binds to classical swine fever virus NS5A and facilitates viral replication

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ABSTRACT

Classical swine fever virus (CSFV) NS5A protein is a multifunctional protein, playing critical roles in viral RNA replication, translation and assembly. To further explore its functions in viral replication, interaction of NS5A with host factors was assayed using a his-tag "pull down" assay coupled with shotgun LC-MS/MS. Host protein translation initiation factor 3 subunit E was identified as a binding partner of NS5A, and confirmed by co-immunoprecipitation and co-localization analysis. Overexpression of eIF3E markedly enhanced CSFV genomic replication, viral protein expression and production of progeny virus, and downregulation of eIF3E by siRNA significantly decreased viral proliferation in PK-15 cells. Luciferase reporter assay showed an enhancement of translational activity of the internal ribosome entry site of CSFV by eIF3E and a decrease in cellular translation by NS5A. These data indicate that eIF3E plays an important role in CSFV replication, thereby identifying it as a potential target for inhibition of the virus.

1. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a notifiable disease of the World Organization for Animal Health (OIE). CSF is a highly contagious swine disease with high morbidity and mortality, causing significant economic losses to the pig industry worldwide. CSF is characterized by hemorrhagic fever and immune suppression. Typical symptoms of disease include petechial bleeding of the skin, mucosae and internal organs, spleen infarction, lymphopenia and granulocytopenia (Thiel et al., 1996; Moennig and Plagemann, 1992; Summerfield et al., 1998). Infection with less virulent strains results in a chronic or subclinical disease with atypical symptoms or inapparent clinical manifestations, with persistently high serum virus titers, but without any detectable humoral or cellular immune responses (Muñoz-González et al., 2015).

CSFV, a member of the genus *Pestivirus* within the family *Flaviviridae* (Simmonds et al., 2011) is a small enveloped virus with a single, positive-stranded RNA genome. The viral genome contains 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 3898 amino acid polyprotein which is co- and post-translationally cleaved by cellular and viral proteases to yield mature structural proteins of C protein, glycoproteins E^{rns}, E1, E2 and 8 nonstructural proteins, N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Tautz et al., 2015).

CSFV NS5A is a phosphorylated and multifunctional protein with 497 amino acids. NS5A regulates viral RNA replication by binding to the 3′-UTR of the viral genome or by modulating NS5B RdRp activity via interaction with NS5B (Sheng et al., 2012a; Chen et al., 2012). NS5A decreases viral IRES-mediated translation in a dose-dependent manner and may be involved in the switch from translation to replication (Xiao et al., 2009), an action that can be suppressed by NS5B protein via binding to NS5A (Sheng et al., 2012b). NS5A is also an essential factor for viral assembly and production, which may be related to its interaction with core protein (Sheng et al., 2014) or with host annexin A2 protein (Sheng et al., 2015). Dong and Tang (2016) reported that NS5A inhibited the secretion of inflammatory cytokines induced by poly(I:C) through suppression of the NF-κB signaling pathway. However, details of the functions of CSFV NS5A in the viral replicative cycle remain to be elucidated.

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Mammalian translation initiation factor 3 (eIF3) is an 800 kDa protein complex, consisting of 13 subunits (a–m). It is the largest, most complex, eukaryotic translation initiation factor and participates in almost all steps of translation initiation (des Georges et al., 2015). Eukaryotic translation initiation begins with formation of a 43S pre-initiation complex by the binding of eIF1, eIF1A and the eIF2–GTP–-Met–tRNAi ternary complex to the ribosome 40S subunit. This subsequently attaches to the cap-proximal region of mRNA after the unwinding of its secondary structure by eIF4A, eIF4B, and then scans the 5′ untranslated region to the initiation codon, where it forms a 48S complex with codon–anticodon base-pairing through eIF5. Finally, eIF5 and eIF5B promote the joining of the 48S complex with a ribosome 60S subunit, forming an elongation-competent 80S ribosome (Jackson et al., 2010; Marchione et al., 2013; des Georges et al., 2015). In this process, eIF3 plays a critical role in the binding of initiator methionyl-tRNA to the 40 S ribosomal subunit, followed by scanning of the mRNA for a start codon to form the 48 S initiation complex (Hinnebusch, 2006; Damoc et al., 2007). At the 5′ UTR, m(6)A mediates cap-independent translation, eIF3 binds m(6)A and participates in recruitment of the 43S complex to initiate translation (Meyer et al., 2015). eIF3 also functions in translation regulation, cell growth and cancer (Marchione et al., 2013). In addition, eIF3 subunits interact with several viral proteins and regulate viral replication (Morais et al., 2013; Komarova et al., 2007; Sato et al., 2007). Here we demonstrate that the eukaryotic translation initiation factor 3 subunit E (eIF3E), associates with CSFV NS5A and promotes viral replication.

2. Results

2.1. Identification of cellular proteins binding to CSFV NS5A protein

Purified His-NS5A or His-tag protein coupled to Dynabeads (Invitrogen, Carlsbad, CA, USA) were incubated with PK-15 cell protein extracts. Proteins specifically binding to His-NS5A or His-tag were visualized by Coomassie brilliant blue (G250) staining (Fig. 1A) and identified by shotgun liquid chromatography-mass spectrometry (LC-MS/MS). Among proteins binding to His-NS5A but not to the His-tag, eIF3E was selected for further investigation because of the known involvement of this eIF3 family member in the replication of other viruses (Morais et al., 2013; Komarova et al., 2007; Sato et al., 2007). The MS data are shown in the Supplementary material.

2.2. Interaction of cellular protein eIF3E with NS5A

To validate the interaction of NS5A with eIF3E as identified by MS analysis, western blot probes of eIF3E co-purifying with fusion protein His-NS5A were conducted using rabbit anti-eIF3E antibody. Fig. 1B shows that eIF3E associated with NS5A rather than the His-tag. Because of its low molecular weight, 0.8 kDa, the His tag protein control was not visible by PAGE or western blot.

Co-IP of NS5A and eIF3E was used to further investigate the interaction between these two proteins. PK-15 cells were transfected with recombinant vectors p3xFLAG-NS5A or p3xFLAG-CMV-10. At 48 h post-transfection, cell lysates were immunoprecipitated with anti-FLAG antibody, and precipitated proteins were subjected to western blotting using anti-eIF3E antibodies. A positive result was observed only in precipitates containing fusion protein FLAG-NS5A (Fig. 1 C). These data further confirm the interaction of eIF3E with NS5A.

2.3. Co-localization of cellular eIF3E with CSFV NS5A in PK-15 cells

The subcellular localization of NS5A and eIF3E within PK-15 cells expressing Flag-NS5A was analyzed by immunofluorescence staining and confocal microscopy. Fig. 2A shows a strong co-localization of both proteins.

2.4. Co-localization of cellular eIF3E with CSFV dsRNA in infected PK-15 cells

PK-15 cells infected with CSFV were fixed and incubated with anti-dsRNA and anti-eIF3E antibodies and the indicated second antibody. Fig. 2B shows the co-localization of eIF3E with dsRNA in CSFV infected cells.

2.5. Inhibition of viral replication by siRNA downregulation of eIF3E

PK-15 cells were transfected with siRNAs targeting eIF3E or with non-targeting control siRNA (NC). Western blot analysis showed that eIF3E expression in PK-15 cells transfected with siRNA-1 (S1), siRNA-2 (S2) or siRNA-3 (S3) separately (Fig. 3A left) or with S1 and S3 together (Fig. 3A right) was significantly decreased compared with untreated cells or cells transfected with NC alone. In addition, MTT assay shows knockdown of eIF3E doesn't result obviously decrease in cell viability (Fig. 7).

At 24 h following transfection with pooled S1and S3, PK-15 cells were infected with virulent CSFV Shimen strain. Viral replication was determined by quantitative analysis of progeny virus, viral genome and viral protein E2 by 24 h, 48 h and 72 h post infection. A significant decrease in viral protein E2 (Fig. 3B), viral genome copies (Fig. 4A) and titers of progeny virus (Fig. 4B) were observed in siRNA treated cells compared with controls.

2.6. Facilitation of CSFV proliferation by overexpression of eIF3E

To upregulate eIF3E, PK-15 cells were transfected with recombinant pDsRed1-eIF3E vectors expressing eIF3E (Fig. 5A). MTT assay shows that overexpression of eIF3E doesn't cause obvious damage to cell viability (Fig. 7). 24 h after transfection, the cells were infected with virulent CSFV Shimen strain. Viral replication was analyzed by virus titration, western blotting of viral E2 protein and quantification of viral genome copies at 24 h, 48 h and 72 h post-infection. Results showed that viral E2 protein (Fig. 5B), viral genome copies (Fig. 6A) and titers of progeny virus (Fig. 6B) in cells with overexpression of eIF3E all increased markedly compared with untreated controls.

2.7. Enhancement of CSFV IRES activity by eIF3E

Since NS5A has been reported to decrease CSFV IRES-mediated translation (Xiao et al., 2009), we investigated whether eIF3E could also affect IRES activity. A bicistronic reporter plasmid pRLuc-IRES-Fluc was used to measure luciferase activity and mRNA level simultaneously under conditions of eIF3E knockdown or overexpression. RLuc and FLuc activity represent cellular cap-dependent translation and CSFV IRES-mediated translation respectively. There was no difference in the rate of luciferase mRNA transcription between RLuc and FLuc, whether overexpression or knockdown of eIF3E (Fig. 8), but knockdown of eIF3E reduced CSFV IRES activity by 43.6% ($p < 0.05$) (Fig. 8) and cellular translation by 23.8% compared to that of NC treated cells, overexpression of eIF3E increased CSFV IRES activity by 110.9% (Fig. 8) and cellular translation by 43.5% compared to that of control cells. These data demonstrate that CSFV IRES-mediated translation is more sensitive to changes in eIF3E expression level than cellular cap-dependent translation and that eIF3E can enhance CSFV IRES- mediated translational efficiency.

2.8. Reduction of cellular translation by NS5A

It is reported that viral protein can inhibit host translation by interaction with eIF3 subunits (Komarova et al., 2007; Sato et al., 2007). Hence inhibition of cellular translation by CSFV NS5A was investigated. 293 cells were transfected with pRL-TK encoding *Renilla* luciferase and increasing amount p3xFLAG-NS5A vector expressing NS5A, luciferase