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Classical swine fever virus NS5A protein interacts with 3′-untranslated region and regulates viral RNA synthesis

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ABSTRACT

To investigate the function of classical swine fever virus (CSFV) NS5A protein, the experiments for viral RNA synthesis and viral replication were performed in the co-presence of NS5A and NS5B. Results showed that small concentrations of NS5A stimulated, large concentrations of NS5A inhibited, viral RNA synthesis and viral replication. Affinity chromatography experiments and UV-crosslinking assays revealed that CSFV NS5A and NS5B bound its cognate 3′UTR and that NS5A had higher affinity than NS5B protein in binding to 3′UTR. 200 ng of NS5A inhibited NS5B–3′UTR complex formation by about 95%. CSFV 3′UTR was found to contain two NS5A-binding sites, located in 3′UTRSL-1 (nt 161–231) and 3′UTRSL-2 (nt 90–160), respectively, a NS5B-binding site, also located in 3′UTRSL-1. The 3′UTRSL-1 is the common binding site for NS5A and NS5B. Furthermore, competitive electrophoretic mobility shift assays indicated that binding of CSFV NS5A to 3′UTRSL-1 is more efficiently than to 3′UTRSL-2. These results suggested that the different concentrations of NS5A, the different binding activities of NS5A and NS5B to 3′UTR and binding of NS5A to different regions of 3′UTR might contribute at least partially to modulation of CSFV replication.

1. Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever, which is a highly contagious and sometimes fatal viral disease of pigs. CSFV, bovine viral diarrhea virus (BVDV 1 and BVDV 2), and border disease virus (BDV) are members of the *Pestivirus* genus within the *Flaviviridae* family (Becher and Thiel, 2002; Heinz et al., 2000). BVDV and BDV can infect both ruminants and pigs. The hepatitis C virus (HCV), the major cause of blood transfusion-associated hepatitis, also belongs to this family (Cuthbert, 1994). HCV shares many molecular and virological similarities with *pestiviruses*. *Pestiviruses* are small, enveloped, positive-strand RNA viruses. The *pestivirus* genome contains a single large ORF, a 5′-untranslated region (5′UTR) and a 3′UTR. The ORF is translated and the resulting polyprotein is subsequently processed into mature proteins by cellular and viral proteases: 4 structural proteins (C, E^{rns}, E1, E2) and 8 nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Moennig and Plagemann, 1992). In its host cell, the single positive strand RNA is transcribed into a negative strand, which serves as a template to produce more positive strand RNAs for packaging into progeny viral capsids (Gong et al., 1996). The 3′UTR and the 5′UTR are thought to regulate *Pestivirus* genome replication (Yu et al., 1999; Isken et al., 2003, 2004; Xiao et al., 2004; Pankraz et al., 2005). An internal ribosome entry site (IRES), located in the 5′UTR, is able to regulate translation of the viral genomes (Fletcher and Jackson, 2002).

The CSFV NS5B protein has RNA-dependent RNA polymerase (RdRp) activity and is able to bind its cognate 3′UTR and initiate genome replication (Steffen et al., 1999; Xiao et al., 2002, 2004). The CSFV NS5A protein comprises 497 amino acids. The exact function of NS5A in the life cycle of CSFV remains unknown. It has been reported that HCV NS5A protein is an essential component of the viral RNA replication machinery and may also function in modulation of the host cell environment (He et al., 2001; Tellinghuisen et al., 2004). Interaction of this protein with core protein is critical for the production of infectious virus (Masaki et al., 2008). BVDV NS5A protein contains an essential zinc-binding site. A zinc-binding site mutation abolishes BVDV RNA replication (Tellinghuisen et al., 2006). In addition, the HCV NS5A plays a role in regulating viral and cellular mRNA translation (He et al., 2003; Kalliampakou et al., 2005; Wu et al., 2008). Mutation analysis in the CSFV genome has indicated the importance of the conserved sequence C2717–C2740–C2742–C2767 in the NS5A for CSFV growth and viral RNA synthesis and the mutations within NS5A can be *trans*-complemented (Sheng et al., 2010). The CSFV NS5A protein has been shown to decrease IRES-mediated translation in a

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dose-dependent manner (Xiao et al., 2009). Recently, HCV NS5A protein has been found to modulate NS5B RdRp activity (Shirota et al., 2002; Quezada and Kane, 2009). To understand the role of CSFV NS5A in viral replication, we investigated the interaction of CSFV NS5A with 3′UTR and found that CSFV NS5A regulated viral RNA synthesis and viral replication and that the modulation was relative to the different binding activities of NS5A and NS5B to 3′UTR.

2. Materials and methods

2.1. Prokaryotic expression and purification of NS5A and NS5B proteins

Prokaryotic expression and purification of NS5A and NS5B proteins were performed as previously described (Xiao et al., 2006). In brief, total RNA was extracted from CSFV Shimen strain. The sequences for a full-length NS5A (amino acids 2684–3180) and NS5B (amino acids 3181–3898) were obtained by RT-PCR, and cloned into the pET28 (a) vectors. Additional sequences coding for six histidines at the C terminus were engineered to facilitate the purification of these proteins. The inserted regions of all clones were sequenced through dideoxynucleotide sequencing and no changes were found. These resulting plasmids were introduced into the *Escherichia coli* strain BL21 (DE3) for expression driven by the bacteriophage T7 RNA polymerase. Expression was induced by addition of isopropyl-D-1-thiogalactopyranoside (IPTG). These proteins were purified using nickel–nitrilotriacetic acid (Ni–NTA)–sepharose resin (Gibco BRL). Protein solutions and dilutions of bovine serum albumin with known concentrations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), transferred onto nitrocellulose membranes, and subjected to Western blot analysis with specific antibodies. Antibodies specific to CSFV NS5A and NS5B were produced by immunization of rabbits with *E. coli*-expressed NS5A and NS5B, respectively, as described previously (Xiao et al., 2006, 2009). The protein amount was determined by densitometry scanning and comparing the two samples on the same gel.

2.2. RNA preparation

The CSFV 3′UTR RNA fragments were prepared as described previously (Xiao et al., 2006). The expected cDNA fragments were cloned into the pGEM-T vector (Promega). After plasmids were extracted and sequenced, the corresponding RNA templates were synthesized by PCR and subsequent *in vitro* transcription. DNA Vent polymerase (New England Biolabs) and a primer containing the bacteriophage T7 promoter were used in the PCR. After the sequence was verified, the resulting PCR products served as templates for the subsequent *in vitro* transcription. The *in vitro* transcription was performed in 50 μl reaction mixtures following the standard method: 20 μl 5× transcription buffer, 2 μl RNasin (20–40 U μl^{-1}) (Promega), 5 μl each NTP (2.5 mM), 5 μg template and 2 μl T7 RNA polymerase (10–20 U μl^{-1}) (Promega). The mixture was incubated at 37 °C for 2 h. Then, 2 μl DNase I (5 U μl^{-1}) (TaKaRa) was added to the mixture and incubated at 37 °C for 15 min. The mixture was extracted with phenol/chloroform. After ethanol precipitation, the RNA was dried and redissolved in 20 μl double distilled H_2O. Integrity of the RNA was analyzed by denaturing formaldehyde/agarose gel electrophoresis. The concentration of RNA was determined by measuring OD_{260}.

2.3. Transfection and preparation of cytoplasmic extracts

CSFV subgenomic replicons, CSM-NS5A$^-$ and CSM, were prepared and transfected into PK15 cells as previously described, respectively (Sheng et al., 2010). CSM is a replicon that carries a deletion of the sequence encoding amino acids 72–79 of the core gene, and that CSM-NS5A$^-$ is replication-defective due to mutation of the first cysteine of the Zn-binding domain of NS5A (Sheng et al., 2010). CSFV NS5A (amino acids 2684–3180), or NS5B (amino acids 3181–3898) was cloned into a pcDNA3.1/N-FLAG vector, or pcDNA3.1 vector and transfected into PK15 cells (Xiao et al., 2009; Wang et al., 2011). Preparation of cytoplasmic extracts from transfected PK15 cells was carried out as previously described with slight modification (Xiao et al., 2002). Approximately 3.0×10^8 cells were pelleted, washed three times with PBS, carefully suspended in a cell lysis buffer (200 mmol/l Tris–HCl pH 8.0, 500 mmol/l NaCl, 10 mmol/l $MgCl_2$, 2% Triton X-100, 10 mmol/l imidazole, and 50% glycerol), and incubated at 4 °C for 30 min. A supernatant was obtained by centrifugation at 10,000 × *g* at 4 °C for 10 min.

2.4. Rescue of viruses and determination of virus growth curves

Rescue of viruses and determination of virus growth curves were performed as described previously (Sheng et al., 2010). The CSFV genomic replicon SM (Sheng et al., 2010), 2 μg, was co-transfected into PK15 cells together with increasing amounts of a pcDNA3.1/N-FLAG-NS5A vector (0.3, 0.5, 0.7, 0.9, 1, 2, 3, 4 μg, respectively). Cell cultures were harvested by freeze–thawing at 48 h after transfection. Cell suspensions were clarified by centrifugation at 5000 × *g* for 10 min at 4 °C. CSFV-specific fragments of the 3′UTR were characterized by RT-PCR. Monolayers were also subjected to an indirect immunofluorescence assay with CSFV-specific E2 mAb F48-3-10 (E. Weiland, Tubingen, Germany) and an FITC-labeled secondary antibody to determine whether viruses were rescued in cells. $TCID_{50}$ ml^{-1} was calculated using the method of Reed and Muench (1938).

2.5. RdRp assays

RdRp assays were performed essentially as described previously (Xiao et al., 2006). Total volume was 50 μl, containing the following supplements: 50 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 10 μM DTT, 25 mM KCl, l mM EDTA, 20 U RNasin, 50 μg/ml actinomycin D (Sigma), 200 μM each NTP, 1 μl of RNA template (250 ng/ml), 100 ng NS5B proteins and increasing concentrations of NS5A (0, 2, 4, 6, 8, 10, 30, 50, 100, 150 and 200 ng, respectively). The mixture was incubated at 37 °C for 1 h, and the reaction was stopped by the addition of 2 μl of EDTA (200 mM). The reaction samples were extracted with phenol/chloroform, and RNAs were precipitated with isopropyl alcohol.

2.6. Quantification of viral RNA

Viral RNA was quantified by real-time RT-PCR (Wang et al., 2011). Forward primer (nucleotides 12,071–12,093, 5′-GCGCGGGTAACCCGGGATCTGAA-3′), reverse primer (12,174–12,190, 5′-CAGTTCTTACTCATTCA-3′), and TaqMan probe (nucleotides 12,103–12,124, FAM-5′-AGGACCCTATTGTAGATAACAC-3′-TAMRA) were designed based on the 3′UTR sequence of CSFV Shimen strain (GenBank AF092448). Total RNA was extracted from the products of RdRp assays with phenol/chloroform. After ethanol precipitation, the RNA was dried, and redissolved in 20 μl of double distilled H_2O. The concentration of RNA was determined by measuring its absorbance at 260 nm. RT was performed using Superscript II reverse transcriptase (Invitrogen). The quantitative real-time PCR was run using the AbiPrism7000 sequence detection system (Applied Biosystems). PCR was performed for 40 cycles with cycling conditions of 15 s at 95 °C and 1 min at 60 °C. *In vitro*-transcribed CSFV RNAs of known