Virus Research 210 (2015) 90–99

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The N-terminus of classical swine fever virus (CSFV) nonstructural protein 2 modulates viral genome RNA replication

Ling Li, Rui Wu, Fengwei Zheng, Cheng Zhao, Zishu Pan*

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China

ARTICLE INFO

Article history:
Received 11 May 2015
Received in revised form 17 July 2015
Accepted 21 July 2015
Available online 29 July 2015

Keywords:
Classical swine fever virus
Nonstructural protein 2
Replication
Replicon

ABSTRACT

Pestivirus nonstructural protein 2 (NS2) is a multifunctional, hydrophobic protein with an important but poorly understood role in viral RNA replication and infectious virus production. In the present study, based on sequence analysis, we mutated several representative conserved residues within the N-terminus of NS2 of classical swine fever virus (CSFV) and investigated how these mutations affected viral RNA replication and infectious virus production. Our results demonstrated that the mutation of two aspartic acids, NS2/D60A or NS2/D60K and NS2/D78K, in the N-terminus of NS2 abolished infectious virus production and that the substitution of arginine for alanine at position 100 (NS2/R100A) resulted in significantly decreased viral titer. The serial passage of cells containing viral genomic RNA molecules generated the revertants NS2/A60D, NS2/K60D and NS2/K78D, leading to the recovery of infectious virus. In the context of the NS2/R100A mutant, the NS2/I90L mutation compensated for infectious virus production. The regulatory roles of the indicated amino acid residues were identified to occur at the viral RNA replication level. These results revealed a novel function for the NS2 N-terminus of CSFV in modulating viral RNA replication.

1. Introduction

Pestiviruses are a group of economically important livestock viruses that are classified as one genus within the family *Flaviviridae*, which also contains the genera *Flavivirus* and *Hepacivirus* (hepatitis C viruses [HCV]). The genus *Pestivirus* encompasses bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV) (Lindenbach et al., 2007). Pestiviruses are single-stranded, positive-sense RNA viruses with genomes of ~12.3 kb that contain one large open reading frame (ORF) encoding a polyprotein of approximately 3989 amino acids that is co- and post-translationally processed into at least 12 mature proteins (Lackner et al., 2004; Lamp et al., 2011 Lamp et al., 2011). Core, E^{rns}, E1 and E2 are the pestivirus structural proteins (Thiel et al., 1991; Weiland et al., 1999), and the nonstructural proteins (NS) NS3 to NS5B are necessary for replication of the viral genome (Behrens et al., 1998; Moser et al., 1999; Risager et al., 2013).

Two additional proteins, p7 and NS2, are located between the E2 and NS3 proteins of pestivirus. p7, a small hydrophobic polypeptide with an apparent molecular mass of 6–7 kDa, is a viroporin protein that is involved in the release of infectious virions and in virulence (Elbers et al., 1996; Gladue et al., 2012; Griffin et al., 2004; Guo et al., 2013; Steinmann et al., 2007). NS2 is a transmembrane protein that contains an auto-protease responsible for *cis*-cleavage at the NS2/3 junction (Guo et al., 2011; Lackner et al., 2004). Temporal regulation of the cleavage event at the NS2/3 junction is of crucial importance for pestivirus replication and pathogenicity (Lackner et al., 2005a, 2004). Previous studies have shown that NS2-3 cleavage efficiency is related to pestivirus biotype (Kummerer and Meyers, 2000; Moser et al., 1999) and that the NS2-3 precursor is required for infectious particle formation (Agapov et al., 2004; Gu et al., 2000; Moulin et al., 2007). However, recent observations have indicated that infectious BVDV is produced in the absence of the NS2-3 precursor and that a critical determinant in the N-terminal portion of NS2 is required for infectious virus production (Lattwein et al., 2012). NS2, although not essential for replication, is involved in regulating pestivirus RNA replication (Moser et al., 1999).

On the molecular level, pestiviruses exhibit striking similarity to an important human pathogen, HCV (Lindenbach et al., 2007). HCV NS2 is composed of a hydrophobic N-terminal membrane binding domain and a C-terminal cytosolic protease subdomain. The protease subdomain can form a dimer with two composite active sites (Lorenz et al., 2006). This protease is not directly required for RNA replication but must be cleaved off of the N-terminus of NS3 to

* Corresponding author. Fax: +86 27 68752833.
E-mail address: zspan@whu.edu.cn (Z. Pan).

http://dx.doi.org/10.1016/j.virusres.2015.07.021
0168-1702/

enable the formation of an active replicase (Welbourn et al., 2005). HCV NS2 is essential for viral particle assembly (Jirasko et al., 2008; Jones et al., 2007 Jones et al., 2007), which occurs independently of protease activity (Dentzer et al., 2009; Phan et al., 2009). The N-terminal portion of NS2 can inhibit the expression of reporter genes driven by a variety of cellular and viral promoters in a dose-dependent manner (Dumoulin et al., 2003), and NS2 can suppress protein expression driven by the HCV internal ribosome entry site (IRES) element, reducing HCV replication (von dem Bussche et al., 2010). In addition, NS2 serves as a scaffold by interacting with both structural and non-structural proteins to recruit E2, NS3 and NS5A to assembly sites in close proximity to lipid droplets (LDs) to regulate infectious virus production (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011). However, the exact mechanism of the multifunctional NS2 in RNA replication and infectious virus production is poorly understood. In this study, we used a combination of biochemical and genetic methods to investigate the role of NS2 in viral RNA replication. We mapped several residues in the N-terminal portion of NS2 that are critical in regulating the CSFV life cycle.

2. Materials and methods

2.1. Cell culture and virus

Porcine kidney 15 (PK15) and swine kidney 6 (SK6) cells were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The PK15 and SK6 cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and nonessential amino acids. The CSFV strain Alfort/187 (vA187) was generated from a cDNA clone (pA187) (Ruggli et al., 1996).

2.2. Construction of CSFV cDNA mutants

A full-length infectious clone, pA187, was used as a template to generate various cDNA mutants. Point mutations were introduced into PCR-amplified fragments by overlapping PCR using specific primers. PCR-amplified fragments were cloned into pA187 using the restriction enzymes *Cla*I and *Kpn*I, resulting in site-directed mutagenesis of cDNA clones of the NS2 gene and producing the following constructs: pA187-NS2/T37A, pA187-NS2/K52A, pA187-NS2/D60A, pA187-NS2/D78A, pA187-NS2/W85A, pA187-NS2/R100A, pA187-NS2/D60K, pA187-NS2/D78K and pA187-NS2/D78E. The construct pA187-NS5B$_{GND}$, an NS5B mutant defective in RNA-dependent RNA polymerase (RdRp) activity (Krieger et al., 2001), was used as a negative control. All mutants were sequenced to confirm their identities. The sequences of both primers and mutants are available upon request.

2.3. Construction of the CSFV replicon and derivatives

To obtain a reporter replicon and derivatives expressing luciferase, a chimeric cDNA clone, pA187-Rluc, which contained the *Renilla* luciferase-2A (Rluc2A) reporter gene, was constructed. The Rluc2A fragment is composed of the first 7 amino acids of the Core protein, followed by the Rluc sequence in tandem with the FMDV 2A peptide. Rluc2A was then inserted into the junction between N^{pro} and Core in-frame by overlapping PCR using specific primers. The PCR-amplified product was cloned into pA187 using the restriction enzymes *Cla*I and *Spe*I to generate the pA187-Rluc plasmid. Then, a monocistronic reporter replicon that included a deletion of the coding region from Core to E1 of the CSFV genome (nucleotides 951-2441 of the CSFV genome), pA187/del(C-E1)-Rluc, was generated by PCR using the pA187-Rluc plasmid as a template and specific primers. A single point mutation within NS2 was introduced into pA187/del(C-E1)-Rluc by PCR-based site-directed mutagenesis. pA187/del(C-E1)/NS5B$_{GND}$-Rluc was used as a negative control.

To construct the bicistronic reporter replicon NS3-NS5B/Rluc, a chimeric fragment containing the coding region deleted from Core to NS2 (nucleotides 906-5168) of the CSFV genome and the internal ribosome entry site (IRES) sequence of encephalomyocarditis virus (EMCV) fused to the site between Rluc2A and NS3 was prepared by overlapping PCR using pA187-Rluc and the plasmid pIRES as templates. Then, the chimeric fragment was digested with *Cla*I and *Nco*I and ligated into *Cla*I/*Nco*I-digested pA187 to generate the replicon NS3-NS5B/Rluc. Similarly, a chimeric fragment containing the coding region from Core to p7 (nucleotides 906-3797) of the CSFV genome and the EMCV IRES fused to the site between Rluc2A and NS2 was constructed. Then, the chimeric fragment was digested with *Cla*I and *Nco*I and cloned into *Cla*I/*Nco*I-digested pA187 to generate the replicon NS2-NS5B/Rluc. The bicistronic replicon NS2-NS5B and its mutant NS2/D60K-NS5B were generated from the replicon NS2-NS5B/Rluc and the NS2/D60K-NS5B/Rluc backbone using primers P 2A-F (5′-AACTTTGACCTTCTCAAGTTGGCCGGAGACG-3′) and P 926-R (5′-TCCACTTGCGCCATCATCAGAGCAACTG-3′). The bicistronic replicon NS3-NS5B was constructed using the replicon NS3-NS5B/Rluc backbone with the primers P 2A-F and P 926-R. All replicons were sequenced to confirm their identities.

2.4. Construction of the NS2-3 eukaryotic expression plasmid and derivatives

To evaluate the effects of single amino acid mutations within the NS2 N-terminus on the auto-processing of the NS2/3 junction site, the eukaryotic expression plasmid pNS23-HA and its derivatives were constructed. Briefly, wt NS2-3 and its mutant fragments were amplified by PCR from pA187 or pA187-NS2 mutants, respectively, using specific primers. The purified PCR products were cloned into the pKH3 vector using the restriction enzymes *Sal*I and *Xba*I to generate pNS23-HA. All plasmids were sequenced to confirm their identities. HA-tagged NS2-3 and its cleaved product NS3-HA were analyzed by western blot using an anti-HA monoclonal antibody (mAb) (Sigma).

2.5. In vitro RNA transcription and virus rescue

In vitro transcription of viral genomic RNA and virus recovery were conducted as previously described (Yang et al., 2012) using a T7 MEGAscript™ kit (Applied Biosystems, Carlsbad, California, USA). Briefly, *Srf*I-linearized wt or mutated NS2 pA187 cDNA clones were used as templates. RNA transcripts were purified using a MEGAclear kit (Applied Biosystems). The integrity of the transcribed RNA was assessed by electrophoresis on an agarose gel, and the RNA concentration was estimated using UV spectrophotometry.

For RNA transfection, 10 μl of Lipofectamine® 2000 (Invitrogen) and 1 μg of RNA were diluted in 100 μl of Opti-MEM, and diluted RNA was added to each tube of diluted lipofectamine. The mixture was incubated at room temperature (RT) for an additional 5 min. PK15 cell monolayers grown in 6-well cell culture plates were washed twice with Opti-MEM and incubated with the RNA transfection mixture for 6 h at 37 °C and 5% CO_2; then, the culture medium containing the transfection mixture was replaced with fresh medium and incubated for 72 h. The cells were then fixed with 50% (v/v) methanol/acetone and stained with an anti-NS3 antibody for immunofluorescence (IF) assay.

For electroporation, the SK6 cells were washed twice with ice-cold phosphate-buffered saline (PBS). Approximately 10^7 cells mixed with 7.5 μg of RNA in 400 μl of ice-cold PBS were transferred