Influence of NS5A protein of classical swine fever virus (CSFV) on CSFV internal ribosome entry site-dependent translation

Ming Xiao, Yujing Wang, Zailing Zhu, Jialin Yu, Lingzhu Wan and Jun Chen

College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, PR China

An internal ribosome entry site (IRES) present in the 5′ untranslated region (UTR) promotes translation of classical swine fever virus (CSFV) genomes. Using an in vitro system with monocistronic reporter RNA containing the CSFV 5′UTR, this study found that CSFV NS5A decreased CSFV IRES-mediated translation in a dose-dependent manner. Deletion analysis showed that the region responsible for repressing CSFV IRES activity might cover aa 390–414, located in the C-terminal half of CSFV NS5A. Triple and single alanine-scanning mutagenesis revealed that the inhibitory effect on CSFV IRES-directed translation mapped to the K399, T401, E406 and L413 residues of NS5A. These important amino acids were also found to be present in the NS5A proteins of bovine viral diarrhea virus (BVDV)-1, BVDV-2, border disease virus and hepatitis C virus, indicating that NS5A may play an important role in the switch from translation to replication in these viruses.

Classical swine fever virus (CSFV) is a member of the genus *Pestivirinae*, which also contains bovine viral diarrhea virus (BVDV)-1, BVDV-2 and border disease virus (BDV) (Becher & Thiel, 2002; Heinz et al., 2000). The genus *Pestivirus* belongs to the family Flaviviridae. Hepatitis C virus (HCV), the major cause of transfusion-associated hepatitis, also belongs to this family (Cuthbert, 1994). CSFV is a small enveloped virus. Its genome is a single, positive-sense RNA, which contains a single large open reading frame (ORF) and 5′ and 3′ untranslated regions (UTRs). The ORF encodes a polyprotein of approximately 3900 aa. The 12 CSFV proteins are organized in the polyprotein in the order NH2-Npro-C-Em1-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Moenning & Plagemann, 1992). The NS5B protein serves as the viral RNA-dependent RNA polymerase (RdRp) (Steifens et al., 1999; Xiao et al., 2002) and contains a conserved GDD motif necessary for catalytic activity (Wang et al., 2007). NS3 is a multifunctional protein possessing serine protease, RNA helicase and nuclease triphosphatase activities, and is important for virus replication and even for promoting viral and cellular translation (Gu et al., 2000; Piccinini et al., 2002; Sheng et al., 2007; Suzich et al., 1993; Warrener & Collett, 1995; Xiao et al., 2008; Xu et al., 1997). The 3′UTR is probably involved in initiation of pestiviral genome replication (Isken et al., 2003, 2004; Pankraz et al., 2005; Xiao et al., 2004; Yu et al., 1999), whilst the 5′UTR contains an internal ribosome entry site (IRES), located between nt 40 and 350 at the 5′ terminus of the genome, which is able to facilitate translation of the viral genome (Fletcher & Jackson, 2002).

NS5A of CSFV strain Shimen comprises 497 aa (aa 2684–3180 of the genome) (Fig. 1a). The function of NS5A in the life cycle of CSFV remains unclear. It has been reported that the NS5A protein of HCV is an essential component of the viral RNA replication machinery and may also function in modulation of the host cell environment (Tellinghuisen et al., 2004, 2006). Interaction of this protein with the core protein is critical for the production of infectious virus (Masaki et al., 2008; Miyanari et al., 2007). In addition, HCV NS5A plays a role in regulating viral and cellular mRNA translation (He et al., 2003; Kalliampakou et al., 2005; Wu et al., 2008).

To investigate the influence of the CSFV NS5A protein on IRES-dependent translation, we constructed a monocistronic reporter RNA containing an entire CSFV 5′UTR and a firefly luciferase (FLuc) ORF in 5′ to 3′ order. In brief, the full-length CSFV 5′UTR (nt 1–373) was cloned into the vector pGEM-T along with a PCR-generated FLuc ORF. The cloned DNA sequence was verified and the resulting plasmid was used as a template for T7-mediated transcription reactions as described previously (Xiao et al., 2006). A capped RNA encoding *Renilla* luciferase (RLuc) was used as an internal control to normalize transfection efficiency. The validity of similar systems in addressing IRES-dependent translation mechanisms has been shown previously (Boni et al., 2005; Shimoike et al., 1999). Total RNA was extracted from CSFV strain Shimen. A full-length NS5A cDNA encoding aa 2684–3180 of the genome (Fig. 1a) was obtained by RT–PCR from CSFV Shimen and cloned into the vector pcDNA-3.1 (Clontech). The
monocistronic reporter RNA (1.0 μg) and the capped RLuc RNA (1.0 μg) were co-transfected into PK15 cells by a liposome-mediated transfection method together with an established pcDNA-NS5A vector that expresses NS5A, as described previously (Xiao et al., 2003). As a control, an unrelated protein, β-galactosidase (LacZ), was expressed in the same vector. PK15 cells were cultivated as described previously (Xiao et al., 2003). Cell extracts were prepared 10 h after transfection. FLuc and RLuc activity was measured using a dual luciferase assay system (Promega) according to the manufacturer's instructions. The results showed that expression of the NS5A protein decreased FLuc activity and that the decrease in FLuc activity correlated with transfection of increasing levels of pcDNA-NS5A (results not shown). These data are consistent with previous reports in which HCV NS5A suppressed HCV IRES-directed translation (Kalliampakou et al., 2005).

Previous reports have shown that the region about 120 aa just upstream of the nuclear localization signal of HCV NS5A plays a crucial role in inhibiting HCV IRES activity (Kalliampakou et al., 2005). To define the region of the CSFV NS5A protein responsible for repressing CSFV IRES activity, deletion mutations were introduced into the CSFV NS5A protein. In brief, three deleted forms of NS5A (deletion of the C-terminal 53, 83 and 108 aa, respectively) were obtained by RT-PCR from the genome of CSFV Shimen: NS5AΔC53 was from aa 2684 to 3127, NS5AΔC83 from aa 2684 to 3097 and NS5AΔC108 from aa 2684 to 3072 of the genome (Fig. 1a). These sequences were engineered with BamHI and XbaI sites and cloned into a pcDNA3.1 vector. PK15 cells were co-transfected with the above two reporter RNAs in combination with pcDNA-3.1 vector containing either one of the CSFV NS5A constructs (wild-type or deleted forms) or the lacZ gene. As shown in Fig. 2(f), expression of NS5AΔC53 caused a strong inhibitory effect on CSFV IRES-directed translation to almost the same degree as that caused by the wild-type NS5A protein (Fig. 2c), and expression of NS5AΔC83 also still suppressed CSFV IRES activity (Fig. 2g). In contrast, expression of NS5AΔC108 exhibited no significant effect on CSFV IRES-directed translation (Fig. 2h), suggesting that the region of the CSFV NS5A protein responsible for repressing CSFV IRES activity might cover aa 390–414 of NS5A (Fig. 1a). Expression of the deleted forms of NS5A was confirmed by Western blotting using the corresponding antibody, and dose-dependent expression of the proteins was observed (Fig. 2d, e).

To map the regions of the CSFV NS5A protein essential for repressing IRES activity more precisely, we performed a comprehensive mutagenesis of aa 390–414 by constructing triple alanine-scanning mutations for the fragment covering aa 390–414 (Fig. 1b). Alanine substitutions were introduced by PCR amplification of the NS5A coding region of the genome of CSFV Shimen using primers containing either one of the CSFV NS5A constructs (wild-type or deleted forms) or the lacZ gene. As shown in Fig. 2(f), expression of NS5AΔC53 caused a strong inhibitory effect on CSFV IRES-directed translation to almost the same degree as that caused by the wild-type NS5A protein (Fig. 2c), and expression of NS5AΔC83 also still suppressed CSFV IRES activity (Fig. 2g). In contrast, expression of NS5AΔC108 exhibited no significant effect on CSFV IRES-directed translation (Fig. 2h), suggesting that the region of the CSFV NS5A protein responsible for repressing CSFV IRES activity might cover aa 390–414 of NS5A (Fig. 1a). Expression of the deleted forms of NS5A was confirmed by Western blotting using the corresponding antibody, and dose-dependent expression of the proteins was observed (Fig. 2d, e).
Fig. 2. Effect of CSFV NS5A protein and its deleted forms on IRES-mediated translation. (a, b) Capped RLuc RNA (1.0 μg) and monocistronic reporter RNA (1.0 μg) containing the CSFV IRES and FLuc-encoding sequence were co-transfected into PK15 cells, together with increasing amounts of vector expressing full-length NS5A (open bars) or LacZ (dotted bars). Cell extracts were prepared 10 h after transfection. FLuc (a) and RLuc (b) activity was measured using a dual luciferase assay system. (c) The effect of NS5A on IRES-directed translation was evaluated by comparing the ratios of FLuc : RLuc, with the ratio in the presence of the LacZ expression vector set at 100 %. (d, e) Expression of NS5A, NS5AΔC53, NS5AΔC83, NS5AΔC108 and LacZ was detected by Western blot analysis (d) and quantitative dot blotting (e). (d) A pcDNA-3.1 expression vector (0.5 μg) containing the corresponding gene was used (Xiao et al., 2003). Lane C represents the control (0.5 μg pcDNA-3.1 vector only). (e) Quantitative dot blotting was performed essentially as described previously (De Lisle, 1991). In brief, increasing amounts of pcDNA-3.1 (0, 0.3, 0.5, 0.7 and 0.9 μg, left to right) containing the corresponding gene were used to transfect PK15 cells, from which the cytoplasmic extracts were obtained (Xiao et al., 2003) and dotted onto a nitrocellulose membrane. Antibodies specific to CSFV NS5A protein and sheep anti-rat LacZ-coupled secondary antibody were used. The blot was quantified by densitometry using a reference curve generated for NS5A and its deletion mutants. Antibodies specific to CSFV NS5A were produced by immunization of rabbits with Escherichia coli-expressed NS5, as described previously (Xiao et al., 2006). A mouse anti-LacZ antibody (Gibco-BRL) was used for the detection of LacZ. (f–h) The effect of the CSFV NS5A deleted forms NS5AΔC53 (f), NS5AΔC83 (g) and NS5AΔC108 (h) on IRES-mediated translation was evaluated as described in (c). Results are shown as means ± SD of at least three independent transfections.
significant effect on CSFV IRES-directed translation, suggesting that the amino acids responsible for repressing CSFV IRES activity were contained in all three regions.

Individual amino acid substitutions were then introduced within the M4, M6 and M8 regions. Mutants M4-1 to M4-3, M6-1 to M6-3 and M8-1 to M8-4 were produced (Fig. 1b). As shown in Fig. 3(b), mutants M4-1, M4-3, M6-2 and M8-3 exhibited no significant effect on CSFV IRES-directed translation. However, the other mutants maintained their strong inhibitory effect on CSFV IRES-directed translation, similar to the wild-type NS5A protein, indicating that residues K399, T401, E406 and L413 of NS5A protein might be crucial for the inhibitory effect of the protein on CSFV IRES-directed translation. Expression of these NS5A mutants was confirmed by Western blotting using corresponding antibody (results not shown).

The influence of several viral proteins such as NS3, NS4A, NS4B, NS5A and NS5B on IRES-dependent translation has been investigated previously. Expression of NS3, NS4A and NS5B proteins did not have any significant effect on the activity of HCV IRES (He et al., 2003). Our previous studies revealed that the NS3 protein could promote CSFV IRES-mediated translation. The CSFV NS5B protein did not significantly affect IRES-directed translation alone, but significantly enhanced the stimulative effect of NS3 protein on IRES-mediated translation (Xiao et al., 2008). It has been reported that HCV NS5A protein downregulates HCV IRES-dependent translation (Kalliampakou et al., 2005). In the present work, it was also found that NS5A has an inhibitory effect on CSFV IRES-dependent translation.

A dose-dependent inhibitory effect of NS5A on IRES-dependent translation might be important for the switch from translation to replication in CSFV and related viruses. It has been found that translation and replication are tightly coupled in some positive-sense RNA viruses (Khromykh et al., 2000; Liang & Gillam, 2001; Myers et al., 2001). This allows the replicase to preferentially replicate the genome from which it was translated, as opposed to defective RNA molecules with egregious mutations. In this way, a high error rate of replication should be avoided (RdRp does not have proofreading capabilities). Furthermore, it has been shown that protein synthesis is more efficient in these coupled replication–translation reactions than in corresponding reactions that utilize pre-synthesized RNA (Ryabova et al., 1994). This means that translation to replication reactions are carried out in a continuous-flow format on the same template at different times. Translation is inhibited when replication occurs because RdRp and the ribosome cannot occupy the same RNA template at the same time, which has been
demonstrated in poliovirus. In this virus, replication begins only after translation has been inhibited, and the viral RdRp cannot replicate viral RNA when it is being translated by the ribosome (Barton et al., 1999; Gamarnik & Andino, 1998). NS5A is thought to be an essential component of the viral RNA replication machinery (Masaki et al., 2008; Tellinghuisen et al., 2004, 2006). It has been demonstrated that the N-terminal domain is required but that the C-terminal domain is not required for the function of NS5A in HCV RNA replication (Tellinghuisen et al., 2008). A region covering aa 236–447 present in the C-terminal domain of HCV NS5A has been found to play a crucial role for the inhibitory activity towards HCV IRES function (Kalliampakou et al., 2005). Our results suggest that the region aa 390–414 located in the C-terminal half of CSFV NS5A is responsible for repressing CSFV IRES activity, corresponding to the region of HCV NS5A causing inhibitory effects on virus translation.

Taken together, we suggest that the NS5A protein might be bifunctional, in the sense that this protein stimulates virus replication and also inhibits virus translation by using N- and C-terminal domains.

Furthermore, this report revealed that K399, T401, E406 and L413 of the NS5A protein in the region involved in repressing IRES activity might be crucial to this inhibitory effect of NS5A on CSFV IRES-directed translation, as mutation of these amino acids destroyed this activity. The implication is that K399, T401, E406 and L413 of the NS5A protein might constitute a crucial site of the domain responsible for repressing CSFV IRES activity. To investigate whether NS5A proteins of other related viruses also contain these important amino acids, complete sequence alignment was performed. The results showed that the NS5A proteins of BVDV-1, BVDV-2, BDV and even many HCV isolates possess conserved residues K, T, E and L in corresponding positions (Fig. 3c), suggesting that these conserved amino acids might be important for repressing IRES activity in these viruses, although this still needs to be confirmed experimentally.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30670445, 30870492), Shanghai Leading Academic Discipline Project (530406), Shanghai Municipal Science and Technology Commission (07DZ12038, 08JC1416900) and Shanghai Municipal Education Commission (08ZZZ66).

References


M. Xiao and others


