Classical swine fever virus NS5B protein suppresses the inhibitory effect of NS5A on viral translation by binding to NS5A

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INTRODUCTION

Classical swine fever is a highly contagious and fatal viral disease of pigs. Classical swine fever virus (CSFV) is the causative agent of the disease. CSFV, bovine viral diarrhea virus (BVDV1 and 2) and border disease virus (BDV) are members of the genus Pestivirus within the family Flaviviridae (Heinz et al., 2000; Becher & Thiel, 2002). Hepatitis C virus (HCV), the major cause of transfusion-associated hepatitis, also belongs to this family (Cuthbert, 1994). Pestiviruses are small, enveloped, positive-strand RNA viruses. The pestivirus genome contains a single large ORF, a 5’UTR and a 3’UTR. The ORF encodes a polyprotein that is subsequently processed into 12 mature proteins by cellular and viral proteases: Npro, C, E’ms, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Moennig & 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. An internal ribosome entry site (IRES), located in the 5’UTR, is responsible for initiation of translation of viral genomes (Fletcher & Jackson, 2002; Pankraz et al., 2005).

The CSFV NS5B protein has an RNA-dependent RNA polymerase (RdRp) activity and is able to bind its cognate 3’UTR and initiate genome replication (Steffens et al., 1999). A highly conserved sequence, GDD, is postulated to be involved in the catalytic activity and metal-ion regulation of the enzyme and is a hallmark of the RdRps of HCV, BVDV and CSFV (Lohmann et al., 1997; Lai et al., 1999; Wang et al., 2007). Furthermore, a nucleotidyltransferase activity was observed with CSFV NS5B, as with HCV and BVDV NS5B (Zhong et al., 1998; Ranjith-Kumar et al., 2001; Xiao et al., 2004). The CSFV NS5A protein comprises 497 aa. The exact function of NS5A in the life cycle of CSFV remains unknown. 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. Interaction of this protein with the...
core protein is critical for the production of infectious virus (Masaki et al., 2008). The BVDV NS5A protein contains an essential zinc-binding site; mutation of this site abolishes BVDV RNA replication (Tellinghuisen et al., 2006). Recently, the HCV NS5A protein has been found to modulate NS5B RdRp activity (Shirota et al., 2002; Quezada & Kane, 2009). In addition, HCV NS5A has been found to play a role in the inhibitory activity towards HCV IRES function (Kalliampakou et al., 2005). Mutation analysis of the CSFV genome has indicated the importance of the conserved sequence C2717-C2740-C2742-C2767 in NS5A for CSFV growth and viral RNA synthesis and that 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 dose-dependent manner (Xiao et al., 2009). Here, we investigated the role of NS5B and NS5A in IRES-mediated translation further and found that NS5A has an inhibitory effect on IRES-mediated translation, the CSFV NS5B protein suppresses this inhibitory effect and the suppression might be related to the interaction of NS5B and NS5A.

RESULTS

**CSFV NS5B protein suppresses the inhibitory effect of NS5A on viral translation**

Previous reports have shown, using an RNA reporter system, that the NS5A protein has an inhibitory effect on IRES-mediated translation (Kalliampakou et al., 2005; Xiao et al., 2009). For further investigation of the role of NS5A in CSFV translation, CSFV subgenomic RNAs CSM-NS5A⁺-NS5B⁻ and CSM-NS5A⁻-NS5B⁻ were produced (Fig. 1a). CSM-NS5A⁺-NS5B⁻ has been shown to produce an active NS5A protein and contains a mutant GDD motif in NS5B so that it does not generate novel RNAs (Sheng et al., 2010). CSM-NS5A⁻-NS5B⁻ cannot form active NS5A or NS5B proteins. PK-15 cells were transfected with CSM-NS5A⁺-NS5B⁻ or CSM-NS5A⁻-NS5B⁻; expression of NS3, NS5A and NS5A mutant was detected by Western blot analysis using corresponding antibodies (Fig. 1b). To compare the translation efficiencies of the two subgenomic RNAs, expression of NS3 was quantified. A higher translation efficiency was detected in cells transfected with CSM-NS5A⁺-NS5B⁻ than transfection of CSM-NS5A⁻-NS5B⁻ (Fig. 1b, lower panel). These data showed that expression of NS5A in the cells reduced viral translation.

In order to examine whether NS5B could affect viral translation, PK-15 cells were co-transfected with the indicated transcripts and expression vectors. Results showed that transfection of CSM-NS5A⁺-NS5B⁻ led to lower translation efficiency than transfection of CSM-NS5A⁻-NS5B⁻, in good agreement with the above experiments. Compared with transfection of CSM-NS5A⁺-NS5B⁻ alone, co-transfection of CSM-NS5A⁺-NS5B⁻ and NS5B expression vector upregulated translation efficiency, but co-transfection of CSM-NS5A⁺-NS5B⁻ and NS5B⁻ expression vector did not. We also observed that co-transfection of CSM-NS5A⁺-NS5B⁻ and NS5B expression vector led to almost the same translation efficiency as transfection of CSM-NS5A⁻-NS5B⁻ alone (Fig. 1d). The results implied that an active NS5B protein might suppress the inhibitory effect of NS5A on viral translation. Furthermore, PK-15 cells were transfected with CSM-NS5A⁺-NS5B⁻ in the presence of increasing amounts of NS5B expression vector. As shown in Fig. 1(e), the NS5B protein, in a dose-dependent manner, reduced the inhibitory effect of NS5A on viral translation.

Next, we examined the regulatory effect of NS5A on IRES-mediated translation in the presence of NS5B protein using an RNA reporter system. Firstly, CSFV NS5A decreased CSFV IRES-mediated translation in a dose-dependent manner when NS5A was present alone (Fig. 2a), consistent with a previous report (Xiao et al., 2009). However, the IRES-mediated translation efficiency was increased when NS5A and NS5B were co-present and the increased translation efficiency correlated with increasing levels of expression of NS5B protein. Almost the same expression level of NS5A was observed (Fig. 2b). Transfection of increasing levels of NS5B expression vector alone and co-transfection of NS5A and NS5B GDD mutant expression vectors did not affect IRES-mediated translation (Fig. 2c, d). All evidence indicated that an active NS5B protein suppresses the inhibitory effect of NS5A on viral translation.

**NS5B suppresses the inhibitory effect of NS5A on viral translation by binding to NS5A**

Our previous work has suggested that the region of aa 390–414 in CSFV NS5A might be responsible for inhibiting CSFV IRES activity (Xiao et al., 2009), which is supported by our present data. The truncated NS5A proteins, with deletion of the C-terminal 53 or 83 aa, still had an inhibitory effect on viral translation the same as that of as their wild-type (WT) partner. However, deletion of the C-terminal 108 aa or of aa 390–414 destroyed the inhibitory activity of NS5A (Fig. 3a, b). HCV NS5B has been found to interact with NS5A (Shirota et al., 2002). In order to investigate whether CSFV NS5B can interact with NS5A and whether the interaction can induce the repression activity of the NS5B protein toward NS5A, glutathione S-transferase (GST) pull-down assays of interaction between NS5B and NS5A were performed. WT or mutant NS5A proteins were mixed with NS5B bound to glutathione-agarose (GST–NS5B). NS5A also was mixed with GST protein alone. Results showed that NS5A, NS5AA53 and NS5AA83 were pulled down with GST–NS5B, but NS5A was not pulled down with the GST protein alone, and neither NS5AA108 nor NS5AΔ390–414 was pulled down with GST–NS5B (Fig. 3c). Furthermore, the data from immunoprecipitation analyses revealed that the truncated NS5A protein with deletion of the C-terminal 53 or 83 aa bound to NS5B, as did WT NS5A (Fig. 3d, lanes 2, 4, 6).
The binding activity was lost when the C-terminal 108 aa or aa 139–414 were removed (Fig. 3d: lanes 8, 10). All of these results implied that the region of aa 139–414 in NS5A is important for NS5B binding. To investigate the NS5B binding site in NS5A more precisely, we used mutant forms of NS5A covering aa 390–414, M1–M8, produced previously by constructing triple and quadruple alanine-scanning mutations (Fig. 4a) (Xiao et al., 2009). GST pull-down and immunoprecipitation analyses revealed that none of these mutants bound NS5B (Fig. 4b, c). All data indicated that aa 139–414 of NS5A are necessary for the interaction between NS5A and NS5B, overlapping with the site responsible for inhibiting CSFV IRES activity. We also found that M4, M6 and M8 did not have an inhibitory effect on viral translation (Fig. 4d), indicating that the corresponding mutant regions of M4, M6 and M8 might be
important for the inhibitory activity of NS5A. To map the specific amino acids for the inhibitory activity of NS5A, we used the mutant forms of NS5A, M4-1–3, M6-1–3 and M8-1–4, produced previously by single alanine-substitution mutations within M4, M6 and M8 (Fig. 5a) (Xiao et al., 2009). GST pull-down and immunoprecipitation analyses revealed that all of these NS5A mutants bound NS5B except for M4-1, M4-3, M6-2 and M8-3 (Fig. 5b, c), suggesting that amino acids K399, T401, E406 and L413 might be important for the NS5A–NS5B interaction. These amino acids have been shown to be necessary for the inhibitory effect of NS5A on CSFV translation (Xiao et al., 2009). Furthermore, four CSFV genomic replicons (SM-K399A, SM-T401A, SM-E406A and SM-L413A), constructed by replacing the corresponding amino acids of NS5A with a single alanine, did not produce any infectious particles (Fig. 6a, b), indicating that these four amino acids of NS5A are essential for virus rescue and infection.

Next, we investigated the specific regions in NS5B involved in NS5A–NS5B interaction. NS5BΔN62, NS5BΔC65, NS5BΔN73 and NS5BΔC82 were produced as described
The results for GST pull-down assays and immunoprecipitation analyses showed that deletion of the N-terminal 62 aa and C-terminal 65 aa from NS5B did not reduce the interaction between NS5A and NS5B substantially, but the N-terminal 73 aa, C-terminal 82 aa and NS5B GDD motifs were necessary for the binding of NS5B to NS5A (Fig. 7a–c). The above results suggested that the regions aa 63–72 and 637–653 in CSFV NS5B are important for NS5A–NS5B interaction. Furthermore, eight mutants (NS5B63R1–R3 and NS5B637R1–R5) were produced by replacing aa 63–72 and 637–653 with three or four continuous alanine residues within the NS5B coding gene (Fig. 8a). GST pull-down and immunoprecipitation analyses revealed that none of these mutants bound NS5A (Fig. 8b, c), indicating that aa 63–72 and 637–653 of NS5B are necessary for the interaction between NS5A and NS5B.

DISCUSSION

Previous reports have indicated that the HCV NS5A protein downregulates HCV IRES-dependent translation (Kalliampakou et al., 2005). Our recent data have shown that CSFV NS5A also decreases IRES-mediated translation in a dose-dependent manner (Xiao et al., 2009). NS4A and NS4B proteins have been observed to inhibit translation from the HCV IRES (Kato et al., 2002). Full-length and
truncated NS3 proteins with an RNA-helicase domain enhance CSFV IRES-directed translation (Xiao et al., 2008). However, all of these observations are derived from data obtained by using RNA reporter systems. To obtain more direct and authentic evidence, we investigated the role of CSFV NS5A in viral translation in the context of genomic RNAs. The results indicated that NS5A has an inhibitory effect on CSFV IRES-mediated translation.

CSFV NS5B has been shown not to affect IRES-directed translation alone, but to enhance the stimulative effect of NS3 on IRES-mediated translation significantly (Xiao et al., 2008). In addition, nucleotidyltransferase activity was observed with CSFV NS5B, as with HCV and BVDV NS5B (Zhong et al., 1998; Ranjith-Kumar et al., 2001; Xiao et al., 2004), which is important for viral replication and translation. Here, we present evidence that CSFV NS5B suppresses the inhibitory effect of NS5A on viral translation. However, the suppression activity is not observed in a CSFV NS5B GDD mutant. The mutation from GDD to GHH at the important motif in NS5B might be lethal for the function and structure of this protein. The palm domain, including the GDD motif, is the catalytic domain; mutations in the palm domain may distort the arrangement of the catalytic residues and thus might not be tolerated (Choi et al., 2006). Mutations within the GDD sequence have been found to abrogate the binding of HCV NS5B to a tumour-suppressor protein (Munakata et al., 2005; McGivern et al., 2009). Indeed, in our present report, no CSFV NS5A was found to bind the NS5B GDD mutant.

CSFV NS5A interacting directly with WT NS5B was observed reciprocally in GST pull-down assays and coimmunoprecipitation analyses. Furthermore, a region from positions 390 to 414 containing amino acids K399, T401, E406 and L413, located in the C-terminal half of CSFV NS5A, was found to be important for interaction between NS5B and NS5A. The four amino acids are necessary for virus rescue and infection. Our present and previous reports consistently show that the above-mentioned region and amino acids overlap with the site responsible for inhibition of IRES-mediated translation by the NS5A protein. The site is located in the region aa 390–414 of the C-terminal half of CSFV NS5A, in which the four amino acids are key. Therefore, we infer that the NS5B protein’s repression activity toward the role of NS5A in...
translation might be obtained by binding to NS5A. When NS5B binds to NS5A and the site responsible for inhibiting IRES activity is blocked, the inhibitory effect of NS5A on IRES-mediated translation is destroyed and, as a result, translation efficiency is upregulated.

The site responsible for inhibiting IRES activity and NS5A–NS5B interaction, located in the C-terminal half of CSFV NS5A, might be important for regulation of replication and translation in CSFV. Reports from HCV have demonstrated that the N-terminal domain is essential, but the C-terminal domain is not required, for the function of NS5A in RNA replication (Tellinghuisen et al., 2008). Our present results, together with previous data from HCV and CSFV, indicate that the C-terminal region of NS5A plays a crucial role in regulation of IRES-mediated translation. In this sense, we assume that NS5A could modulate virus replication by using its N-terminal domain and regulate viral translation by using its C-terminal region. In positive-sense RNA viruses, NS5B RdRp does not have a proofreading activity; thus translation and replication are tightly coupled to avoid a high error rate (Khromykh et al., 2000; Liang & Gillam, 2001; Myers et al., 2001). The level of viral translation must be tightly regulated in order to keep up with the level of virus replication; translation is reduced at too high a replication level, and increased at too low a level. This is important for understanding the role of NS5A in the virus life cycle.

**METHODS**

**Transfection and preparation of RNA transcripts and protein expression vectors.** CSFV subgenomic RNA transcripts were prepared from CSFV Shimen strain (GenBank accession no. AF092448) as described previously (Sheng et al., 2010). CSM- and CSM-NS5A+NS5B were generated through in-frame deletion of a region encoding aa 240–247 of the core protein in the complete genome RNA replicons SM- and SM-NS5B, respectively. CSM-NS5A+NS5B is the CSM-transcript with a mutant GDD motif in NS5B, produced through substitution of DD with HH at positions 3628 and 3629 at the amino acid level. CSM-NS5A+NS5B was generated through a substitution of C with A at position 2717 (amino acid level) in NS5A in the context of CSM-NS5A+NS5B (Fig. 1a).

Mutant CSFV genomic replicons SM-K399A, SM-T401A, SM-E406A and SM-L413A were produced by replacing the corresponding amino acids of NS5A with a single alanine in the context of genomic replicon SM- (Sheng et al., 2010).

To construct protein expression vectors, NS5A cDNA encoding aa 2684–3180 and NS5B cDNA encoding aa 3181–3898 of the genome were obtained by RT-PCR from CSFV Shimen strain and cloned into the vector pcDNA-3.1 (Clontech) (Xiao et al., 2003a, 2009). The cDNA sequence encoding NS5B GDD mutant (designated NS5B+) was based on RNA replicon SM-NS5B+. Three deleted forms of NS5A were obtained by RT-PCR from the genome of CSFV.
Shimen: NS5AA53 from aa 2684 to 3127, NS5AA83 from aa 2684 to 3097 and NS5AA108 from aa 2684 to 3072 of the genome. For expression of NS5Adel390–414, aa 390–414 was deleted from WT NS5A (Fig. 4a). A double-stranded site-directed mutagenesis kit (TaKaRa) and the oligonucleotide sites were used and allowed production of the expected mutations in the cloned gene. The inserted regions of all clones were sequenced through dideoxynucleotide sequencing and no changes were found.

Transfection of subgenomic and genomic RNAs or protein expression vectors was carried out with a suspension of PK-15 cells, following the instruction manual for Lipofectamine 2000 (Gibco) as described previously (Sheng et al., 2010). At 10 h post-transfection, expression of NS3, NS5A, NS5B and mutant NS5A (designated NS5A2) was detected by Western blot analysis using NS3-specific, NS5B-specific and NS5A-specific antibodies, respectively (Xiao et al., 2006, 2009; Sheng et al., 2007). Blots were stripped and reprobed with anti-β-tubulin antibody (Sigma-Aldrich). The proteins were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Signal intensities were quantified using Kodak ID 3.5 software.

Northern blotting. Total cellular RNA was extracted from PK-15 cells at 10 h post-transfection using TRIzol reagent (Invitrogen). The RNA samples were analysed by denaturing formaldehyde/agarose gel electrophoresis. Northern blots were probed with a digoxigenin-labelled RNA probe that recognized the CSFV 5’UTR. Northern blotting was performed as described previously (Sheng et al., 2010). The 5’UTR NS5B transcript was detected by Northern blot analysis using NS3-specific, NS5B-specific and NS5A-specific antibodies, respectively (Xiao et al., 2006, 2009; Sheng et al., 2007). Blots were stripped and reprobed with anti-β-tubulin antibody (Sigma-Aldrich). The proteins were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Signal intensities were quantified using Kodak ID 3.5 software.

Construction of monocistronic plasmids, RNA preparation and luciferase assay. Construction of monocistronic plasmids was performed as described previously (Xiao et al., 2011). In brief, the IRES DNA sequence (nt 60–393) was amplified by RT–PCR from the genome of CSFV strain Shimen and inserted into the pGL3-Basic vector (Promega), in frame with the encoding sequence of Fluc. The IRES–Fluc DNA sequence was cloned into the pGEM-T Easy vector (Promega) to generate pGEM/IRES/Fluc. A pGEM/CAP/Rluc plasmid containing an Rluc sequence controlled by a 5’ cap structure from pRL-SV40 (Promega) was constructed as described previously (Xiao et al., 2011). Monocistronic reporter RNA and IRES RNA sequences were synthesized by in vitro transcription with the PCR products from pGEM/IRES/Fluc and pGEM/CAP/Rluc, as described previously (Xiao et al., 2011). The concentration of RNA was determined by measuring A260. Finally, monocistronic reporter RNAs containing Fluc under the control of the IRES and the capped Rluc RNA were produced (Fig. 3a). The capped Rluc RNA (1.0 μg) and the monocistronic reporter RNAs (1.0 μg) containing the CSFV IRES and Fluc-encoding sequences were co-transfected into PK-15 cells in the presence or absence of NS5A (WT or mutants) and/or NS5B (WT or mutants) expression vector. Cell extracts were prepared 10 h after transfection. Fluc and Rluc activities were measured using a dual luciferase assay system (Promega) according to the manufacturer’s instructions.

Prokaryotic expression and purification of proteins. Prokaryotic expression of CSFV NS5A and its truncated forms were performed as described previously (Xiao et al., 2006). In brief, the cDNAs encoding WT and truncated NS5A were obtained by PCR from their corresponding pcDNA-3.1 expression vectors. Mutant forms of NS5A covering aa 390–414, M1–M8, were expressed as described
previously (Fig. 7a) (Xiao et al., 2009). The cDNA sequences encoding the NS5B GDD mutant (GDD to GHH, designated NS5B \( ^2 \)) were amplified by PCR from subgenomic RNA, CSM-NS5A\( ^+\)-NS5B\( ^2 \). The sequences were cloned into pET28(a) vectors. A His\(_6\) tag was added to the C terminus of each of these proteins to facilitate purification. The inserted regions of all clones were sequenced through dideoxynucleotide sequencing and no changes were found. NS5B and NS5B truncated forms NS5B\( ^{D\_N62} \), NS5B\( ^{D\_C65} \), NS5B\( ^{D\_N73} \) and NS5B\( ^{D\_C82} \) were expressed as described previously (Xiao et al., 2003b, 2006). A comprehensive mutagenesis of aa 63–72 and 637–653 of NS5B was performed by constructing triple or quadruple alanine-scanning mutations for the corresponding fragments. Alanine substitutions were introduced by PCR amplification of the NS5B coding region of the genome of CSFV Shimen using primers containing the desired changes.

The resulting plasmids were introduced into *Escherichia coli* strain BL21(DE3) for expression driven by T7 RNA polymerase. Expression was induced by addition of IPTG. The bacterial cell culture was harvested by centrifugation at 6000 \( g \) for 10 min and washed in PBS.

The cleared lysates were obtained by centrifugation at 35 000 \( g \) for 15 min and then purified using nickel–nitrilotriacetic acid (Ni–NTA)–Sepharose resin (Gibco-BRL) as described previously (Wang et al., 2011). Dilutions of BSA with known concentrations were also subjected to SDS-PAGE. The gels containing the samples were stained with Coomassie brilliant blue. The amount of each of these proteins was determined by densitometry scanning and comparing the two samples on the same gel.

**GST pull-down assay.** GST–NS5B and GST–NS5A fusion proteins were expressed as described previously (Wang et al., 2010). In brief, the cDNA sequences encoding WT NS5B and NS5A were amplified by PCR and inserted into a pGEX-4T-1 vector (Amersham Pharmacia Biotech), in frame with the GST-encoding sequence. GST, GST–NS5B or GST–NS5A fusion protein was expressed in *E. coli* and purified with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) (Dimitrova et al., 2003). Expression was induced by addition of IPTG. Cells from a 500 ml culture were then harvested and sonicated in 45 ml PBS buffer supplemented with a protease inhibitor.
cocktail and 1% Triton X-100. Insoluble materials were pelleted at 15,000 g for 10 min, and 500 μl of a 50% slurry of glutathione–Sepharose 4B beads was added to the clarified supernatants. The beads were allowed to bind proteins for 1 h, washed three times in PBS and finally resuspended in 500 μl PBS supplemented with 1% Triton X-100.

GST pull-down assays were performed as described previously (Wang et al., 2011). Approximately 2 μg GST, GST–NS5B or GST–NS5A fusion protein immobilized on 20 μl GST resin was pre-blocked with 1% BSA and then incubated with 2 μg NS5A (WT or mutants) or NS5B (WT or mutants) in 300 μl PBST buffer (PBS containing 1% Triton X-100) for 3 h on a rotating device at room temperature. After washing with PBST, the bound proteins were fractionated by SDS-PAGE (10% acrylamide) and subjected to Western blot analysis with anti-NS5A or anti-NS5B antibody.

**Immunoprecipitation analysis.** Immunoprecipitation analysis was performed as described previously (Wang et al., 2011). For immunoprecipitation analyses, the sequences encoding NS5A (WT and mutants) and NS5B (WT and mutants) were obtained by PCR from the pET28(a) vectors containing these protein-encoding regions described above for prokaryotic expression, cloned into a pcDNA3.1/N-FLAG vector. The inserted regions of all clones were sequenced through dideoxynucleotide sequencing and no changes were found. The pcDNA3.1/N-FLAG/NS5A(WT and mutant) vector, together with the pcDNA3.1 vector containing NS5A, were used for co-transfection of PK-15 cells by using Lipofectamine 2000. The cells were harvested at 48 h post-transfection, washed and sonicated in RIPA buffer consisting of 150 mM NaCl, 0.5% Triton X-100, 10 mM Triton/HCl (pH 7.5). Lysates were centrifuged at 12,000 g for 10 min at 4°C and the supernatants were immunoprecipitated with anti-NS5A antibody or anti-NS5B antibody for 1 h at 4°C, followed by incubation with 20 μl protein G–agarose beads (Pharmacia) at 4°C for 1 h. After washing with RIPA buffer, the bound proteins were eluted, fractionated by SDS-PAGE (10% acrylamide), transferred onto nitrocellulose membranes and subjected to Western blot analysis with anti-FLAG mAb, anti-NS5B antibody or anti-NS5A antibody. Protein bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

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**Fig. 8.** (a) Schematic of the mutant forms of NS5B covering aa 63–72 and 637–653. Positions of aa 1, 63, 72, 637, 653 and 718 are shown over the NS5B protein. The NS5B mutant forms are shown under aa 63–72 and 637–653. Names of WT and mutant NS5B proteins are shown on the left. Dotted lines represent the rest of the NS5A sequence. Amino acids replaced by triple and quadruple alanines are underlined. (b, c) GST pull-down (b) and immunoprecipitation (c) analysis of interaction between CSFV NSSA and the indicated mutants, as described in the legend to Fig. 4(b, c), respectively.
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