A positively charged surface patch on the pestivirus NS3 protease module plays an important role in modulating NS3 helicase activity and virus production

Fengwei Zheng1 · Weicheng Yi1 · Weichi Liu2 · Hongchang Zhu1 · Peng Gong2 · Zishu Pan1

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Abstract
Pestivirus nonstructural protein 3 (NS3) is a multifunctional protein with protease and helicase activities that are essential for virus replication. In this study, we used a combination of biochemical and genetic approaches to investigate the relationship between a positively charged patch on the protease module and NS3 function. The surface patch is composed of four basic residues, R50, K74 and K94 in the NS3 protease domain and H24 in the structurally integrated cofactor NS4APCS. Single-residue or simultaneous four-residue substitutions in the patch to alanine or aspartic acid had little effect on ATPase activity. However, single substitutions of R50, K94 or H24 or a simultaneous four-residue substitution resulted in apparent changes in the helicase activity and RNA-binding ability of NS3. When these mutations were introduced into a classical swine fever virus (CSFV) cDNA clone, a single substitution at K94 or a simultaneous four-residue substitution (Qua_A or Qua_D) impaired the production of infectious virus. Furthermore, the replication efficiency of the CSFV variants was partially correlated with the helicase activity of NS3 in vitro. Our results suggest that the conserved positively charged patch on NS3 plays an important role in modulating the NS3 helicase activity in vitro and CSFV production.

Introduction
Pestiviruses are causative agents of economically important livestock diseases [10, 15, 21, 37]. Members of the genus Pestivirus include classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV). The pestivirus genome consists of a single positive-stranded RNA of approximately 12.3 kb, with one open reading frame (ORF) flanked by a 5′ untranslated region (UTR) and a 3′ UTR. The ORF encodes a polyprotein of approximately 4,000 amino acids [2, 8, 25]. The polyprotein is co- and post-translationally processed by viral and host proteases to produce 12 mature proteins [5, 14, 16, 17], including four structural proteins (C, Ems, E1 and E2) [38, 41] and eight nonstructural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [12, 18, 27, 40].

NS3 has serine protease and RNA helicase/nucleotide triphosphatase (NTPase) activity, and both are essential for virus replication [13, 27, 36, 42, 43]. Circumstantial evidence suggests that the protease and helicase/NTPase domains of NS3 are functionally interdependent [26, 29]. NS3 protease activity requires NS4APCS as a structurally integrated cofactor [36, 49]. In the case of hepatitis C virus (HCV), the NS3 protease domain enhances its helicase activity [3, 4]. The HCV NS3 helicase activity is modulated by NS5B, an RNA-dependent RNA polymerase (RdRP), and the protease domain is required for the interaction between NS3 and NS5B [1, 48]. In the case of CSFV, a truncated NS3 protein (NS3Hel) containing only the helicase domain has been shown to exhibit similar NTPase activity and significantly decreased helicase activity when compared to the full-length NS3 (NS3fl) [33, 39, 42, 43], thereby further demonstrating that the protease and helicase domains of NS3
A

5’UTR  Npro  C  E1  E2  P  NS2  NS3  NS4A  NS4B  NS5A  NS5B  3’UTR

NS4APCS

Helicase

Protease

R50
K74
K94
H24

CSFV

···LKIRGIQG···VTCKDKKV···QSNKMTDE···GYQALSRRHIPVVTD···

BVDV

···LKVRGIQG···VTAGDKKV···QSNKLTD···GYQALSRRVPMITD···

BDV

···LKIRGIQG···VTAGDKKV···QSNKMTDE···GYQALSRRVPMITD···

NS3 Protease

NS4APCS

B

Pro

H24
K74
K94

Hel

C

Marker  WT  NS3 hel

130 kD  100 kD  70 kD  55 kD  35 kD

Set 1

Set 2

H24A  R50A  K74A  K94A  Quad_A

H24D  R50D  K74D  K94D  Quad_D

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are functionally coupled. However, the exact mechanism of this intramolecular regulation needs to be addressed further.

Our previous study revealed an intramolecular protease-helicase interface with a positively charged groove in the pestivirus NS3 structure. Four basic residues (R50, K74 and K94 in NS3 and H24 in NS4APCS) in the protease part of the groove form a positively charged surface patch, which maybe coordinate with the helicase part of the groove to modulate the RNA-binding ability and helicase activity of NS3 [49]. In this study, we further investigated the relationship between this positively charged surface patch and NS3 function. Our data suggest that, in this natural protease-helicase fusion protein, the positively charged surface patch of NS3 plays an important role in modulating NS3 helicase activity and infectious virus production.

Materials and methods

Plasmid construction

To prepare the wild-type (WT) NS3 and its mutated proteins, two sets of NS3 constructs containing a single substitution or simultaneous substitution of the four basic residues (R50, K74 and K94 in the protease domain and H24 in NS4APCS) in the positively charged surface patch to alanine or aspartic acid (Fig. 1A) were generated by using an NS3 expression plasmid (pET28a-NS3S163A/NS4APCS) as a template, using a QuikChange Site-Directed Mutagenesis Kit [49, 50]. The variants generated by mutating the basic residue to alanine (A) or aspartic acid (D) are referred to as set 1 or set 2, respectively. When the four basic residues were simultaneously replaced by alanine or aspartic acid, the variant was correspondingly named Quad_A (Quadruple_A) or Quad_D (Quadruple_D). A helicase-only construct (NS3Hel, residues 204 to 683) and a variant harboring a K232A mutation in NS3 to abolish its ATPase and helicase activities were used as negative controls. We also introduced the mutations of both sets into a full-length CSFV infectious clone [20] as described previously [45] to investigate the effect of the basic residue substitutions on infectious virus production. All variants were confirmed by sequencing.

Protein expression and purification

The expression and purification of NS3S163A/NS4APCS and its variants were performed as described previously [22, 49]. Briefly, E. coli strain BL21-CodonPlus (DE3)-RIL was transformed with the expression plasmid, and the bacteria were then cultured at 37°C in terrific broth (TB) medium containing 50 μg of kanamycin and 25 μg of chloramphenicol per ml. When the optical density at 600 nm (OD600) of the culture reached 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After an additional incubation at 25 °C for 4 h, the cells were harvested for subsequent experiments.

The harvested cells were resuspended in a lysis buffer (150 mM Na2SO4, 50 mM Tris [pH 8.0], 10 mM imidazole, 0.02% [wt/vol] NaN3, 20% [vol/vol] glycerol) and then lysed using an AH-2010 homogenizer (ATS Engineering Ltd.) at 14,500 lb/in². After centrifugation, the clarified lysate was loaded onto a HisTrap HP column (GE Healthcare), and the target protein was eluted with an elution buffer (300 mM imidazole, 50 mM Tris [pH 8.0], 150 mM Na2SO4, 20% [vol/vol] glycerol, and 0.02% [wt/vol] NaN3). The protein fractions were pooled, concentrated, and passed through an ENrich SEC 650 column (Bio-Rad) equilibrated with 150 mM NaCl, 5 mM Tris 7.5, 10% (vol/vol) glycerol, and 0.02% (wt/vol) NaN3. The concentrated protein was flash frozen in liquid nitrogen and stored as aliquots at – 80 °C. The protein concentration was determined by the Bradford method [6, 35].

ATPase assay

The ATPase activity was measured using a malachite-green-based method as described previously [39, 43, 49]. Briefly, the 90-μl reaction mixture except for the ATP substrate was incubated at 37 °C for 5 min. The reaction was initiated by addition of a 10-μl ATP solution to yield a final reaction mixture containing 10 nM NS3, 50 mM Tris (pH 7.5), 2.5 mM MgCl2, 50 mM NaCl, and 5 to 500 μM ATP. After an additional incubation at 37 °C for 15 min, the malachite green mixture (water, 0.081% [wt/vol] malachite green, and 5.7% [wt/vol] ammonium molybdate in 6 M HCl at a ratio of 3:2:1 [vol:vol:vol]) was added, and the absorbance was immediately measured at 630 nm on a Multiskan MK3 microplate reader (Thermo Fisher Scientific). Initial ATPase catalytic rates were determined based on the slope of the initial absorbance change and the reference standard curve of absorbance versus phosphate concentration determined independently. The observed ATP hydrolysis rates at various
ATP concentrations were fitted to Michaelis-Menten kinetics to yield the ATPase parameters ($K_m^{app}$ and $k_{cat}$).

### Helicase assay

A helicase unwinding pssRNA substrate T40:R20 was prepared by annealing the template strand (T40, 5′-GGGCCA AUCAUCAGAA CAGAUCUAACCU-C3′) and the release strand (R20, 5′-UACUGUAUG CAUGAUUGG-3′) labeled with 3′-6-TAMRASE (6-carboxy-tetramethylrhodamine N-succinimidyl ester) [49]. A typical 20-µl unwinding reaction mixture contained 8 U of RNasin (RiboLock; Thermo Scientific), 50 mM morpholinepropanesulfonic acid (MOPS)-NaOH (pH 7.0), 5 mM ATP, 2.5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 0.5% Tween 20, 0.1 mg of bovine serum albumin per ml, 10 nM T40:R20 (according to the concentration of the R20), 100 nM unlabeled release strand (compeitive strand), and 25 nM NS3 (protein/pssRNA molar ratio = 2.5:1). The unwinding reaction proceeded at 37 °C for 30 min and was terminated by addition of 2.2 µl of a 10× loading buffer (50 mM Tris [pH 7.5], 50 mM EDTA, 1% [wt/vol] SDS, 50% [vol/vol] glycerol, and 0.1% [wt/vol] xylene cyanol). RNAs in the quenched reaction mixtures were resolved by 12% nondenaturating polyacrylamide gel electrophoresis. The fluorescent signal of 6-TAMRASE-labeled R20 was detected using a Molecular Imager PhorosFX$^\text{TM}$ Plus System (Bio-Rad) with an excitation wavelength of 532 nm and a 605 nm emission filter. The band intensities were quantified using ImageJ (http://imagej.nih.gov/ij), and the percentage of unwound RNA was calculated based on the fluorescence intensity of the released R20. Each unwinding reaction was performed independently four times.

### Fluorescence polarization (FP)‑based RNA binding assay

To measure the RNA-binding ability of NS3 and its variants, we designed pssRNA-2 by annealing the template strand (T40, 5′-GGGCCA AUCAUCAGAA CAGAUCUAACCU-C3′) and the release strand (R20, 5′-UACUGUAUG CAUGAUUGG-3′) labeled with 3′-6-TAMRASE (6-carboxy-tetramethylrhodamine N-succinimidyl ester) [49]. A typical 20-µl unwinding reaction mixture contained 8 U of RNasin (RiboLock; Thermo Scientific), 50 mM morpholinepropanesulfonic acid (MOPS)-NaOH (pH 7.0), 5 mM ATP, 2.5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 0.5% Tween 20, 0.1 mg of bovine serum albumin per ml, 10 nM T40:R20 (according to the concentration of the R20), 100 nM unlabeled release strand (compeitive strand), and 25 nM NS3 (protein/pssRNA molar ratio = 2.5:1). The unwinding reaction proceeded at 37 °C for 30 min and was terminated by addition of 2.2 µl of a 10× loading buffer (50 mM Tris [pH 7.5], 50 mM EDTA, 1% [wt/vol] SDS, 50% [vol/vol] glycerol, and 0.1% [wt/vol] xylene cyanol). RNAs in the quenched reaction mixtures were resolved by 12% nondenaturating polyacrylamide gel electrophoresis. The fluorescent signal of 6-TAMRASE-labeled R20 was detected using a Molecular Imager PhorosFX$^\text{TM}$ Plus System (Bio-Rad) with an excitation wavelength of 532 nm and a 605 nm emission filter. The band intensities were quantified using ImageJ (http://imagej.nih.gov/ij), and the percentage of unwound RNA was calculated based on the fluorescence intensity of the released R20. Each unwinding reaction was performed independently four times.

### Virus rescue and titration

The virus was rescued as described previously [20, 45, 46]. Briefly, PK-15 cells were transfected with 2 µg of the WT CSFV cDNA clone or its variants containing two sets of mutations, using Lipofectamine 3000 (Invitrogen). After incubation at 37°C for 72 h, virus production was monitored by indirect immunofluorescence assay (IFA) using an anti-NS3 rabbit polyclonal antibody as the primary antibody [20] and an Alexa Fluor 488-conjugated secondary antibody (goat anti-rabbit IgG, Invitrogen). The culture supernatant was harvested and clarified by centrifugation, and virus titration was performed by IF staining [20] with anti-NS3 antibody in 96-well plates using the Reed-Muench method [32]. Virus titers were expressed as tissue culture infectious doses (50% endpoint, TCID$_{50}$) per milliliter.

### RT-qPCR

Viral RNA copy numbers were determined using a reverse transcription quantitative PCR (RT-qPCR) [19, 30]. PK-15 cell monolayers in 24-well plates were infected with the virus at an MOI of 0.001. Total RNA was extracted from the infected cells at 6, 12, and 24 hpi using a TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa) and 500 ng of total RNA was reverse transcribed using a ReverTra Ace qPCR RT Kit (TaKaRa), with a specific primer (5′-TAG CCTAAATAGTGCCCTCTG-3′). Then, the cDNA transcribed from 50 ng of total RNA was analyzed by qPCR using a THUNDERBIRD Probe qPCR Mix kit (TaKaRa) with a 5′-FAM-labeled probe (5′-TCAGTGCTACTCCC ATCACGTTGTTGA-3′) and the primers CSFV cDNA clone or its variants containing two sets of mutations, using Lipofectamine 3000 (Invitrogen). After incubation at 37 °C for 30 min, polarization was monitored using a Cytation 3 Cell Imaging Multi-Mode Reader (Bio Tek) by exciting at 485 nm (20 nm bandwidth) and measuring total fluorescence intensity and parallel and perpendicular polarized light at 528 nm (20 nm bandwidth). The G-factor (the instrument calibration factor) was calculated using readings from wells with 10 nM pssRNA-2 alone. The data at different NS3 concentrations ([S]) were fitted to the quadratic equation $f = A x [(K_d + [S] + 10)^2/4 - [S] 	imes 10]$, where the “A” represents the amplitude of the FP value change, 10 is the concentration of pssRNA-2 (10 nM), and $K_d$ is the dissociation constant of the NS3-RNA binding complex.

RT-qPCR

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(log_{10} copies/μg) were calculated from three independent experiments.

**Statistical analysis**

Statistical analysis was performed using Student’s t-test. A p-value less than 0.05 was considered significant.

**Results**

**Expression and purification of NS3 and its mutants**

To investigate the effect of amino acid substitutions in the positively charged surface patch of NS3 on enzyme activities, expression plasmids harboring a single substitution or simultaneous substitution of four basic residues (H24, R50, K74 and K94) (Fig. 1) were constructed using the plasmid pET28a-NS3/NS4APCS as a template [49]. These basic amino acids are highly conserved in pestivirus NS3 proteins (Fig. 1A, bottom). The four basic amino acids were simultaneously changed to alanine (A) or aspartic acid (D) in the NS3/NS4APCS surface patch to generate the corresponding mutant, Quad_A or Quad_D. Truncated NS3 (NS3Hel) and the mutant NS3/K232A were used as controls in enzyme assays [18, 42]. The recombinant proteins were expressed in E. coli, purified, and analyzed by SDS-PAGE (Fig. 1C).

**The positively charged surface patch modulates the helicase activity of NS3 independently of its ATPase activity in vitro**

To assess the effect of basic amino acid substitutions in the NS3/NS4APCS surface patch on the helicase and ATPase activities of NS3, we first measured the ATPase activity of WT NS3 and its mutants in vitro. ATP hydrolysis by NS3 (WT) and its mutants (for simplicity, hereinafter collectively referred to by the mutated amino acid symbol) were determined under steady-state conditions, and the data were fit to the Michaelis-Menten equation (v = V_{max}[S]/K_{M} + [S]) to calculate the apparent K_{M} (K_{M}^{app}) and k_{cat} by non-linear regression using GraphPad Prism software. The data showed that the mutants containing the substitutions at conserved sites in the NS3 basic patch had K_{M}^{app} (ATP) values in the range of 35-56 μM and k_{cat} values in the range of 4.4-5.5 sec^{-1}. For the controls, the K_{M}^{app} (ATP) of WT was 42.2 ± 2.4 μM and k_{cat} was 5.1 ± 0.1 sec^{-1}, and NS3Hel had a K_{M}^{app} (ATP) of 36.6 ± 3.6 μM and k_{cat} of 4.4 ± 0.1 sec^{-1} (Fig. 2A and Table 1). These results indicate that perturbation of the positively charged surface patch of NS3 had no influence on its ATPase activity.

We also measured the RNA helicase activity of NS3 and its mutants at a saturating ATP concentration. A partially single-stranded RNA (pssRNA) T40:R20 was used as a helicase substrate. The percentage of the unwound release strand relative to the total amount of release strand was calculated based on the fluorescent signals from native polyacrylamide gel electrophoresis (PAGE) separating free R20 from the T40:R20 complex. WT NS3 unwound 80% of the R20 under the tested conditions, while NS3Hel unwound only 15% of the substrate (Fig. 2B and Table 1). For the mutant NS3/K232A (NC), an extremely low unwinding activity was observed (Fig. 2B, 6% unwound). In set 1, the R50A, K74A, and K94A mutants unwound 71%, 86%, and 64% of the substrate, respectively. The mutants H24A and Quad_A exhibited a significantly decreased unwinding activity (Fig. 2B, 50% and 54% unwound) compared to WT NS3. In set 2, the mutant K74D unwound 71% of the substrate, and the unwinding activities of the remaining mutants significantly decreased compared to WT NS3 (Fig. 2B; 47-64% unwound). The mutant Quad_D had the lowest helicase activity (Fig. 2B). These observations collectively suggest that the basic residues in the NS3 surface patch modulate the helicase activity in vitro.

**The basic residues in the surface patch regulate the RNA-binding ability of NS3 synergistically**

To further investigate the mechanism by which the charged patch in the NS3 protease domain regulates RNA unwinding activity, we assessed the RNA-binding ability of NS3 and its mutants. We hypothesized that the 5' region of the substrate release strand resides at the back of NS3 and allows itself to bind the charged patch on the protease domain. We prepared another pssRNA-2 by annealing the T40 template strand and a 5'-FAM-labeled 33-mer release strand (R33). An FP-based RNA binding assay [28, 31] showed that the apparent equilibrium dissociation constant (K_{d}) of NS3 was 30.4 ± 4.3 nM and that the mutant NS3/K232A (NC) exhibited similar RNA-binding ability, with a K_{d} of 39.4 ± 9.9 nM (Fig. 3 and Table 1). The truncated mutant NS3Hel bound very little of the RNA substrate, and the value of K_{d} could not be detected under the tested conditions (Fig. 3 and Table 1). In set 1, the mutant Quad_A exhibited a significantly higher K_{d} value, 84 nM, but the K_{d} values of remaining mutants ranged from 24 to 39 nM, similar to NS3 (Fig. 3 and Table 1). In set 2, the mutant Quad_D had a K_{d} value of 160 nM, and the remaining mutants had K_{d} values in the range of 27-58 nM (Fig. 3 and Table 1). Compared to Quad_A, Quad_D exhibited significantly decreased RNA-binding ability. These results suggest that the four basic residues in the protease domain regulate the helicase activity by synergistically affecting the RNA-binding ability of NS3.
Fig. 2 The ATPase and helicase activities of CSFV NS3 and its mutants. (A) ATPase kinetics curves. ATPase activity was measured using a malachite-green-based method. The initial reaction rates at different ATP concentrations were fitted to a standard Michaelis-Menten curve (mean ± SD; n = 3). Ctr, control. (B) The helicase activity was measured using a T40:R20 pssRNA substrate. Annealed, denatured, boiled T40:R20 (lower, the released R20); Ctr, NC, NS3Hel, WT; set 1, the mutants harboring alanine substitutions; set 2, the mutants harboring aspartic acid substitutions. Measurement of helicase activity was carried out independently four times, and the percentage of mean unwound RNA is shown below the gels.

Table 1 Effects of charged amino acid substitutions on the NS3/4A PCS surface on enzymatic and viral characteristics

| Construct  | Helicase activity (%) | ATPase activity | $K_M$ (nM) | Viral antigen | Virus titer (log_{10} TCID_{50}/ml) | Viral RNA copies (per µg of total RNA) |
|------------|-----------------------|----------------|-----------|--------------|-----------------------------------|----------------------------------------|
|            |                       | $K_M^{app}$ (µM) | $k_{cat}$ (s⁻¹) |              |                                   |                                        |
| WT NS3     | 79.5 ± 9.3            | 42.2 ± 2.4     | 5.1 ± 0.1 | 30.4 ± 4.3   | 7.4 ± 0.3                         | 2.9 ± 0.5 4.1 ± 0.5 5.9 ± 0.4            |
| NS3Hel     | 15.1 ± 4.9            | 36.6 ± 3.6     | 4.4 ± 0.1 | –            | –                                 | ND                                     |
| NC         | 5.7 ± 0.8             | ND             | ND        | 39.4 ± 9.9   | –                                 | ND                                     |
| H24A       | 50.4 ± 10.2           | 56.3 ± 3.2     | 4.8 ± 0.1 | 23.7 ± 7.9   | 6.4 ± 0.4                         | 3.1 ± 0.1 4.0 ± 0.1 5.0 ± 0.1           |
| R50A       | 71.5 ± 6.5            | 40.5 ± 2.8     | 4.8 ± 0.1 | 39.3 ± 8.4   | 5.7 ± 0.5                         | 2.7 ± 0.5 3.8 ± 0.5 5.1 ± 0.4           |
| K74A       | 85.6 ± 2.1            | 38.6 ± 4.1     | 5.1 ± 0.2 | 26.8 ± 7.1   | 7.4 ± 0.4                         | 3.0 ± 0.6 4.2 ± 0.6 5.9 ± 0.6           |
| K94A       | 63.7 ± 5.0            | 45.8 ± 4.2     | 4.8 ± 0.1 | 38.1 ± 9.7   | –                                 | ND                                     |
| Quad_A     | 54.3 ± 10.0           | 35.5 ± 3.3     | 5.5 ± 0.1 | 84.3 ± 18.3  | –                                 | ND                                     |
| H24D       | 64.2 ± 9.6            | 35.9 ± 4.5     | 5.3 ± 0.2 | 49.1 ± 12.4  | 2.6 ± 0.5                         | 2.6 ± 0.4 3.6 ± 0.4 4.0 ± 0.1           |
| R50D       | 59.5 ± 9.3            | 34.6 ± 3.4     | 5.5 ± 0.1 | 32.0 ± 9.5   | 5.9 ± 0.4                         | 2.8 ± 0.2 3.5 ± 0.2 4.4 ± 0.1           |
| K74D       | 70.9 ± 5.3            | 42.3 ± 4.1     | 4.9 ± 0.1 | 57.8 ± 13.1  | 4.5 ± 1.1                         | 2.6 ± 0.6 3.0 ± 0.3 4.2 ± 0.3           |
| K94D       | 58.4 ± 7.3            | 38.5 ± 4.0     | 5.0 ± 0.1 | 27.1 ± 8.8   | –                                 | ND                                     |
| Quad_D     | 47.2 ± 4.0            | 41.6 ± 2.8     | 5.0 ± 0.1 | 160.0 ± 33.4 | –                                 | ND                                     |
The positively charged surface patch of NS3 plays an important role in regulating CSFV production

To investigate the role of the positively charged surface patch on NS3 in infectious virus production, we introduced each of the mutations into an infectious full-length cDNA clone of the CSFV Shimen strain, pSPTI/SM [20]. PK-15 cells were transfected with the full-length cDNA construct to rescue infectious CSFV. The infectious rescued virus corresponding to each construct is signified by a lowercase ‘v’ preceding the construct name. The recovery of infectious CSFV was detected by IFA using an anti-NS3 antibody at 72 h post-transfection. The data showed that PK-15 cells transfected with a construct harboring the H24A, R50A, K74A, H24D, R50D, or K74D mutation were positive for viral antigen. No viral antigen was detected in PK-15 cells transfected with an infectious cDNA clone containing the K94A, Quad_A, K94D, or Quad_D mutation (Fig. 4A and Table 1).

Next, we determined the titers of the rescued CSFV mutants. The mutant vK74A had a virus titer similar to that of the wild-type virus vWT. However, the titers of the mutants vH24A, vR50A, vH24D, vR50D, and vK74D were significantly lower (Fig. 4A). Consistent with the viral antigen detection results, no infectious CSFV was rescued when using the K94A, Quad_A, K94D or Quad_D construct (Fig. 4A and Table 1). Viral RNA copy numbers in infected cells were determined using RT-qPCR [19, 30]. The viral RNA copy numbers were significantly lower for the mutants vR50A (6 to 24 hpi), vR50D and vK74D (12 and 24 hpi), and vH24A and vH24D (24 hpi) compared to
vWT. As expected, the levels of genomic RNA were found to be similar for vK74A and vWT (Fig. 4B). The differences in the RNA copy numbers were consistent with those of the virus titers. These results suggest that the positively charged surface patch of CSFV NS3 plays an important role in regulating infectious virus production by modulating viral RNA replication.

Discussion

Although NS3 of the members of the family Flaviviridae is a natural fusion protein with two separable enzymatic modules, the N-terminal protease and C-terminal NTPase/helicase of the protein are functionally coupled. The pestivirus NS3 protease domain catalyzes cleavage of host and viral proteins and is essential for the process of viral RNA replication [36, 49]. The protease domain is required for RNA unwinding by NS3 helicase and greatly enhances the ability of NS3 to bind RNA. Intermolecular electrostatics in HCV NS3 plays an important role in this process [3]. For West Nile virus NS3, this crosstalk between the protease and helicase modules has an autoregulatory function [7]. Perturbation of the CSFV NS3 protease-helicase interface by point mutations impairs the helicase activity in vitro as well as virus production in vivo [49].

It has been widely reported that the NS3 protease domain of members of the family Flaviviridae stimulates the RNA unwinding activity of its helicase [1, 23, 39, 43, 44, 48, 49]. In HCV, NS3Hel has greatly decreased helicase activity and RNA-binding ability compared to NS3fl [3, 11]. The electrostatics and allosteric contribution from the interaction interface between the CSFV NS3 helicase and protease modules play an important role in the enhanced RNA-binding ability of NS3 by the protease domain [49]. Here, we further addressed the effect of residue substitutions in the basic patch on infectious virus production. Interestingly, no direct correlation was observed between NS3 helicase activity and virus production. Unexpectedly, when the residue K94 was mutated to alanine or aspartic acid, no infectious CSFV was rescued although the mutants K94A and K94D still had moderate helicase activity in vitro. Previous studies have demonstrated that HCV NS3 interacts with NS2 and other viral proteins to form a replication complex, which is essential for virus replication and viral particle assembly [24, 34, 47]. Mutations at the interface between the BVDV NS3 protease domain and the NS4A-kink region impairs the NS3/4A-kink interaction, and the mutant is no longer capable of viral RNA replication [9]. We speculated that K94 on NS3 surface patch is essential for replication complex formation, independently of its helicase activity or RNA-binding ability. The precise mechanism of action of the residue K94 on NS3 surface patch needs to be addressed further. Among the remaining mutants with a single amino acid substitution, the virus titer was to a certain extent related to the helicase activity and RNA-binding ability of NS3. The simultaneous four-residue substitutions (Quad_A or Quad_D) resulted in significantly decreased RNA-binding ability and helicase activity of NS3 in vitro. As expected, no infectious virus could be rescued from the full-length cDNA clone harboring the Quad_A or Quad_D mutations. Collectively, our results suggested that the positively charged surface patch on the pestivirus protease module plays an important role in modulating NS3 helicase activity and virus production. These findings contribute to our understanding of the functional regulation of pestivirus NS3 and will be of potential use for the design of novel antiviral strategies.

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Declarations

Conflict of interest

The authors declare that they have no competing financial interests.

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