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Short communication

N-terminus of *Classical swine fever virus* strain *TD96* glycoprotein E^{rns} contains a potential heparin-binding domain

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ABSTRACT

Classical swine fever virus (CSFV) envelope glycoprotein E^{rns} has been shown to bind to cell surface sulphated-heparin-like glycosaminoglycans (GAGs), which participate in cell attachment of the virus. In this study, the CSFV E^{rns} gene was codon optimized for expression in the yeast *Pichia pastoris*. A C-terminally truncated E^{rns} recombinant protein lacking the previously identified heparin-binding domain (HBD) bound to heparin column, suggesting the presence of another HBD in CSFV E^{rns}. Sequence analyses of the CSFV E^{rns} coding region revealed a common potential N-terminal HBD at residues 301 - 311. Site-directed mutagenesis of the basic amino acids at K^{303} and K^{306} significantly reduced the heparin-binding affinity of the protein. Further mutations of both T^{310} and H^{311} had little effect. Thus, a novel potential heparin-binding site near the N-terminus of CSFV strain TD96 E^{rns} has been detected, and the two basic amino acids K^{303} and K^{306} are crucial for binding activity to heparin matrix and cell-surface GAGs.

1. Introduction

Classical swine fever (CSF), caused by *Classical swine fever virus* (CSFV), is an economically important swine disease worldwide. Together with *Bovine viral diarrhoea virus* (BVDV) of cattle, CSFV belongs to the genus *Pestivirus* in the family *Flaviviridae* (King et al., 2011). The *Pestivirus* genome is a positive-stranded RNA molecule that encodes a polyprotein, which ultimately yields all the final viral proteins through its cleavage by both host and viral proteases (Zhang et al., 2011). Its structural proteins include a nucleocapsid protein C and three envelope glycoproteins, E^{rns}, E1, and E2.

CSFV E^{rns} glycoprotein composes with 227 amino acids at positions 268–494 of the virus polyprotein and forms as a homodimer (Meyers and Thiel, 1996). It has been identified as an RNase (Schneider et al., 1993), but the biological function of its RNase activity is not fully understood. E^{rns} protein lacks a typical transmembrane anchor and is secreted from infected cells (RÜmenapf et al., 1993). Heparin is a highly sulfated polysaccharide, highly abundant on the cell surfaces of animals. A large number of viruses utilize heparin to mediate their attachment and infection of their target cells (Chen et al., 1997). CSFV E^{rns} (Hulst et al., 2001) and BVDV E^{rns} (Iqbal et al., 2000) bind to glycosaminoglycans (GAGs) on the cell surface in an early step in the viral infection of cells. The binding of viral proteins or glycoproteins to GAGs via heparin binding domains (HBDs) has been demonstrated, with HBDs having an over-all positively charged sequences (Capila and Linhardt, 2002). A cluster of basic amino acids ^{480}KKLENKSK^{487} near the C-terminus of BVDV E^{rns} is essential for heparin binding, and the two basic lysine residues, K^{481} and K^{485}, are critical to this binding activity (Iqbal and McCauley, 2002). In the present study, the full-length CSFV strain TD96 E^{rns} protein (N227) and a C-terminally truncated mutant (N190) were successfully expressed in the yeast *Pichia pastoris*. Both recombinant proteins could be purified with heparin column chromatography, suggesting the presence of other heparin-binding sites apart from the previously identified C-terminal HBD in BVDV E^{rns}. Sequences analyses of the CSFV E^{rns} coding region revealed a potential N-terminal HBD at residues 301 - 311. The effects of residues important in heparin binding were further examined with site-directed mutagenesis.

2. Materials and methods

2.1. Construction of recombinant expression plasmids

The gene fragment encoding E^{rns} (GenBank accession number AY4397.1) of CSFV TD96 strain which was isolated in 1996 in southern Taiwan was codon optimized (Fig. 1) for expression in *P. pastoris* and synthesized by GenScript Inc. (Piscataway, NJ, USA). The optimized full-length E^{rns} gene fragment encoding 227 amino acid residues was subcloned into the yeast secreting expression vector pGAPZαC to generate the recombinant plasmid pGAPZαC/N227. Recombinant E^{rns} protein was fused an N-terminal peptide encoding from *Saccharomyces cerevisiae* α-factor secretion signal. The gene fragment encoding the N-terminal 190

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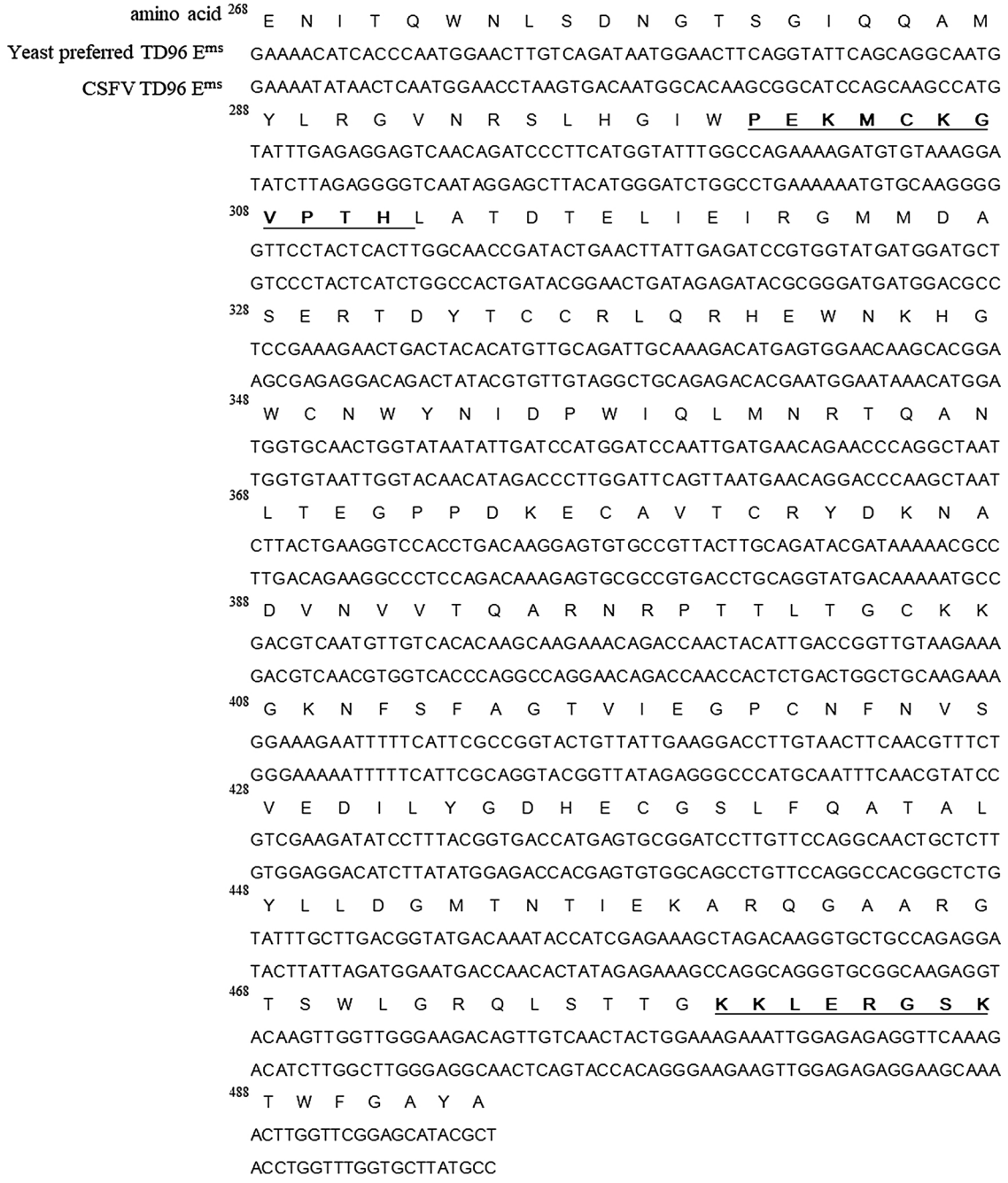

Fig. 1. Sequence comparison of the native CSFV E^{rns} gene and the *Pichia pastoris* codon-optimized E^{rns} gene. Potential heparin-binding domains are underlined.

residues of E^{rns} was prepared with polymerase chain reaction (PCR) using the primer N1F (5′–AAATCGATGGAAAACATCACCCAATG–3′) and N190R (5′–AATCTAGATTAGATGGTATTTGTCATACC–3′), and was then subcloned into pGAPZαC to generate the recombinant plasmid pGAPZαC/N190.

2.2. Expression of E^{rns} proteins in yeast

Each recombinant plasmid was linearized with *Avr*II digestion and introduced into competent *P. pastoris* SMD1168 cells using the *Pichia* EasyComp™ Transformation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The recombinant yeast strains were selected and confirmed with a colony PCR as described previously (Wu et al., 2017). YPD medium (5 ml) was inoculated with a single colony of recombinant yeast and incubated at 30 °C in a shaking incubator (250 rpm) overnight. An aliquot (1 ml) of the overnight culture was then transferred into 50 ml of fresh YPD medium in a 250-ml baffled flask and incubated continuously for 3 days. The supernatants were clarified by centrifugation (20 min, 10,000 × *g*, 4 °C) and the secreted proteins were analyzed with western blotting using a swine immuneserum specific for CSFV.

2.3. Heparin affinity chromatography

The yeast-expressed recombinant E^{rns} proteins were purified with affinity chromatography using a Heparin HP (HiTrap™) column (GE Healthcare). The protein samples were buffer-exchanged into binding buffer (10 mM sodium phosphate) and 2 ml of sample was loaded on the column. The column was washed twice with 5 ml of binding buffer and eluted in 3 ml fractions with a stepwise gradient of increasing concentrations of NaCl (0.1 - 1 M) in binding buffer. The fractions were analyzed with western blotting, and then the specific E^{rns} signals were analyzed with ImageJ software to quantify the eluted E^{rns} protein.

2.4. Site-directed mutagenesis

The critical amino acid residues K^{303}, K^{306}, T^{310}, and H^{311} in the potential N-

Table 1
Comparison of the potential heparin-binding domains of E^{rns} protein of classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV).

Virus Strain (GenBank no.)	N-terminal Sequences (298-315)	C-terminal Sequences (479-494)
CSFV TD96 (AY554397)	GIWPE**K**MC**K**GPV**TH**LATD	G**KK**LER**G**S**K**TWFGAYA
CSFV LPC (AF352565)	GIWPG**K**IC**K**GVP**TH**LATD	G**KR**LEG**R**S**K**TWFGAYA
CSFV 94.4 (AY646427.1)	GIWPE**K**IC**K**GVP**TH**LATD	G**KR**LER**R**S**K**TWFGAYA
CSFV yanshi (ART84242.1)	GIWPE**K**IC**K**GPV**TH**LATD	G**KK**LER**G**S**K**TWFGAYA
CSFV YRSYNE0 (AEI88326.1)	GIWPE**K**IC**K**GPV**TH**LATD	G**KK**LER**G**S**K**TWFGAYA
CSFV LOM/2008 (EU789580.1)	GIWPE**K**IC**K**GVP**TY**LATD	G**RR**LEG**R**S**K**TWFGAYA
CSFV YC16CS (KY290453.1)	GIWPE**K**IC**K**GVP**TH**LATD	G**KK**LER**G**S**K**TWFGAYA
CSFV Alfort/187 (X87939.1)	GIWPE**K**IC**K**GVP**TY**LATD	G**KR**LEG**R**S**K**TWFGAYA
CSFV Brescia (AF091661.1)	GIWPE**K**IC**K**GVP**TY**LATD	G**KR**LEG**R**S**K**TWFGAYA
CSFV CSF0849 (AFP54155.1)	GIWPE**K**IC**K**GPV**TH**LATD	G**KK**LER**G**S**K**TWFGAYA
CSFV Riems (U45477.1)	GIWPG**K**IC**K**GVP**TH**LATD	G**KR**LEG**R**S**K**TWFGAYA
BVDV1 NADL (NC_001461)	GIWPE**K**IC**T**GVP**SH**LATD	G**KK**LEN**K**S**K**TWFGAYA
BVDV1 XZ01 (MF278651)	GIWPE**K**IC**T**GVP**SH**LATD	G**KK**LEN**K**S**K**TWFGAYA

Potential heparin binding domains are underlined and the critical amino acid residues are bold.

terminal HBD were specifically mutated to uncharged alanine residues with PCR using KOD-plus polymerase (Toyobo), according to the manufacturer's instructions. The mutant N227/mKK and N190/mKK mutants were mutated at both $K^{303}$and K^{306}, and the mutant N227/mKKTH was mutated at K^{303}, K^{306}, T^{310}, and H^{311}. All the desired mutations were confirmed with nucleotide sequencing.

2.5. RNase activity assay

The RNase activity of each expressed E^{rns} recombinant protein degrading polymeric RNA substrate was measured by perchloric acid precipitation, and conducted according to Wu et al. (2011) as described in the previous study. Each recombinant E^{rns} protein was incubated with 80 μg of polyU RNA homopolymer in 100 μl renaturing buffer at 37°C for 1 h. The reaction was stopped by adding 100 μl of 1.2 M perchloric acid-25 mM lanthanum sulfate. After 1 h incubation on ice and 15 min of centrifugation (15,000 × g, 4°C), the A260 of the supernatant was measured by spectrophotometer (Hitachi U-2800 A). Each reaction was tested in triplicate, and the optical density (O.D.) of triplicates was averaged.

2.6. Cell-surface binding assay

Confluent PK-15 cells in 96-well plates were washed twice with PBS and fixed with 10% formalin solution (Fisher) at room temperature for 10 min. After the cells were washed with PBS, they were blocked overnight at 4 °C with blocking buffer (PBS containing 3% bovine serum albumin). The cells were incubated with a specific recombinant E^{rns} protein in PBS at room temperature for 1 h. After the cells were washed three times with PBS, the bound recombinant E^{rns} protein was detected with swine immuneserum specific for CSFV in an enzyme-linked immunosorbent assay (ELISA). The relative E^{rns} binding activity was calculated as the absorbance value and the average absorbance for full-length N227 was deemed to be 100%.

2.7. E^{rns} interfering CSFV infectivity assay

One hundred microgram of each E^{rns} recombinant protein was mixed with 200 $TCID_{50}$ CSFV TD96 followed by addition to PK-15 cells and incubation at 37°C for 2 h. The mixture solution was removed and the cells were incubated at 37°C for 3 days. The cells were fixed with 10% formalin solution (Fisher) and infection of CSFV was detected by the monoclonal antibody (WH303) specific to viral E2 in an ELISA. The relative infection ration was calculated as the absorbance value and the average absorbance for DMEM control was deemed to be 100%. Each reaction was tested in triplicate.

3. Results

3.1. Expression of recombinant E^{rns} and mutants in yeast

Codon-optimized DNA fragments encoding the full-length protein (N227)

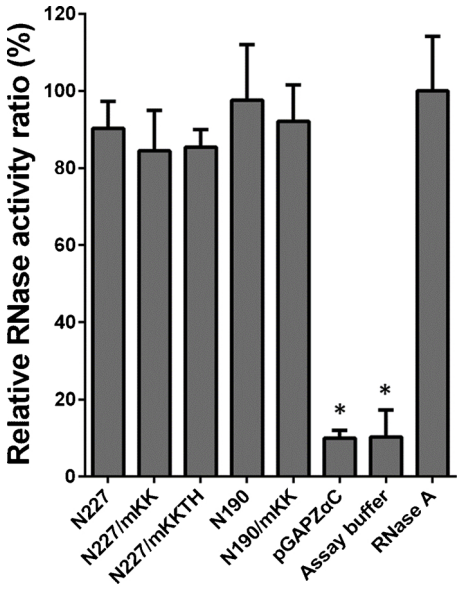

Fig. 2. Analysis of RNase activity of E^{rns} and mutants. Yeast-expressed E^{rns} recombinant protein and mutants were coincidently analyzed to determine their poly U RNA-degrading activity. The pGAPZαC-transformed recombinant yeast strain and RNase A were used as the negative and positive controls, respectively. The bars show the relative RNase activity as a percentage when using absorbance of RNase A as 100%. Asterisks indicate significant differences at $*p < 0.05$.

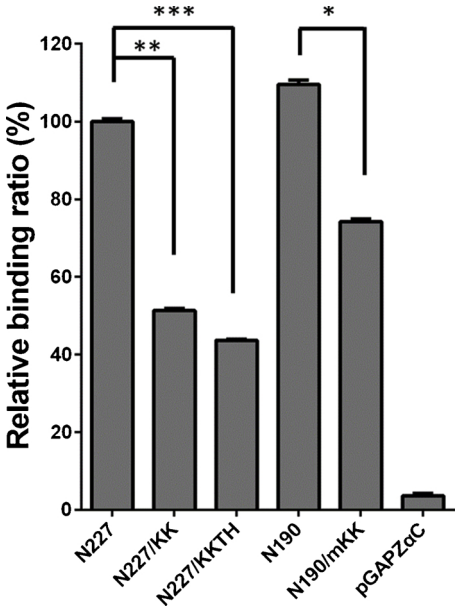

Fig. 4. Binding of E^{rns} and mutants to PK-15 cells. Cell-surface binding of the wild-type and mutant E^{rns} proteins was examined. The pGAPZαC-transformed recombinant yeast strain was used as the negative control. Each bar represents the binding activity relative to that of the wild-type N227. Asterisks indicate significant differences at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

and the C-terminally truncated mutant (N190) were cloned onto the pGAPZαC secreting expression vector for expression in *Pichia pastoris*. Both expressed E^{rns} recombinant proteins N227 and N190 could bind to heparin resin in spite of the N190 lacking the C-terminal HBD previously identified in BVDV E^{rns}. Sequence analyses of different strains of CSFV E^{rns} coding region using NCBI protein blast revealed a common N-terminal potential heparin binding domain at positions 301 - 311 (Table 1). The critical residues including two conserved lysine residues (K^{303} and K^{306}), T^{310}, and H^{311} were mutated to neutral residues by site-direct mutagenesis for further detection of the heparin biding activity. RNase activity analysis of E^{rns} and mutants demonstrated no significant difference among E^{rns} recombinant proteins and positive control RNase (Fig. 2). C-terminal truncation (N190) and those site-mutagenized mutants (mKK and mKKTH) all retained their RNase activity.

3.2. Heparin-binding affinity of recombinant E^{rns} and mutants

The recombinant E^{rns} proteins were purified on a heparin-Sepharose column and eluted with a stepwise gradient of increasing concentrations of NaCl. The wild-type N227 eluted at 0.2 - 0.4 M NaCl, whereas the mutant N227/mKK eluted at 0.1 - 0.2 M NaCl. The C-terminally truncated N190 protein eluted at 0.2 - 0.3 M NaCl, whereas the mutant N190/mKK bound loosely to the heparin matrix and was predominantly detected in the flow-through fraction (Fig. 3).

3.3. Effect of E^{rns} mutation on its binding to the cell surface

The relative cell-binding activities of E^{rns} mutants to PK-15 cells were examined. As shown in Fig. 4, wild-type N227 and the C-terminally truncated mutant N190 showed similar binding to PK-15 cells, but mutations at both $K^{303}$and K^{306} (N227/mKK, N190/mKK) significantly reduced the binding ability of the mutant. Further mutation at T^{310} and H^{311} (N227/mKKTH) only slightly reduced binding activity of the mutant relative to that of N227/mKK.

3.4. Inhibition of CSFV infection by recombinant E^{rns}

The recombinant E^{rns} and mutants were tested for their ability to inhibit virus infection by coinfection with CSFV TD96 to PK-15 cells. As shown in Fig. 5, addition of the full-length N227 or C-terminal truncation N190 during the period of virus attachment decreased CSFV infectivity. Contrastingly, E^{rns} mutants with mutation at both K^{303} and K^{306} showed no inhibitory effect on virus infection.

4. Discussion

The CSFV glycoprotein E^{rns} is a highly glycosylated molecule, with seven potential N-glycosylation sites at positions 269, 275, 278, 332, 362, 410, and 425 of the virus polyprotein (RÜmenapf et al., 1993). N-glycosylation of E^{rns}

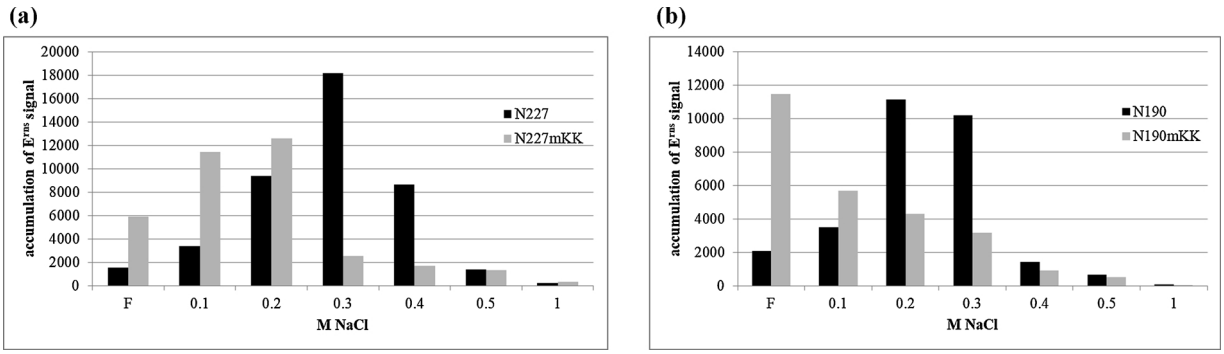

Fig. 3. Binding activities of E^{rns} proteins to heparin-Sepharose. Recombinant E^{rns} proteins N227 and N227mKK (a), N190 and N190mKK (b) were applied to a heparin-Sepharose column, and the E^{rns} proteins were eluted with a stepwise NaCl gradient (0.1 - 1.0 M). The fractions, including flow-through (F) and each eluted fraction, were examined with western blotting followed by an ImageJ analysis.

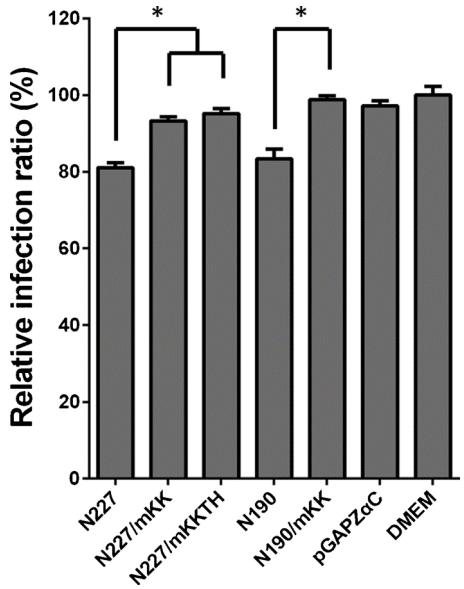

Fig. 5. Effects of E^{rns} and mutants on CSFV infectivity. The abilities of E^{rns} and mutants to inhibit virus infection were examined by addition of each recombinant protein or DMEM during the CSFV infection of PK-15 cells. The bar shows the relative infection ratio of coinfection with each recombinant protein when using absorbance of DMEM as 100%. Asterisks indicate significant differences at $*p < 0.05$.

play an important role in secretory pathway (Branza-Nichita et al., 2004). N269 A/Q substitution to remove a N-glycosylation site caused the virus attenuation that produced a transient infection in swine characterized by mild symptoms and decreased virus shedding (Sainz et al., 2008). The yeast *P. pastoris* has become a popular expression system for producing important glycoproteins because of its low cost, ease of manipulation, posttranslational processing systems, and potential for large-scale production (Cereghino and Cregg, 2000; Wu et al., 2017). Codon optimization is an effective strategy for enhancing the expression of recombinant proteins in appropriate host (Cheng et al., 2014). In this study, the E^{rns} gene of the currently epidemic CSFV strain TD96 was codon optimized for the yeast *P. pastoris* expression system. The full-length E^{rns} protein (N227) and C-terminally truncated mutant (N190) were successfully expressed. Interestingly, the N190 protein lacking the C-terminal HBD which has been previously identified in BVDV E^{rns} at residues 480 - 487, (Iqbql and McCauley, 2002), bound to heparin resin, suggesting the presence of another HBD site. Sequence analyses of the E^{rns} genes from different CSFV strain revealed conserved potential HBD sites at each terminus (Table 1). At the C-terminal HBD (^{480}KKLENKSK^{487}), CSFV LPC strain (genotypes 1) and 94.4 strain (genotype 3) retain the same two critical basic amino acids at residues 481 and 485 as those identified in BVDV. However, the residue at position 485 in strain TD96 (genotype 2.1) is neutral (glycine). The binding affinities of full-length N227 and C-terminally truncated N190 were similar (Fig. 3), implying that the C-terminal HBD of strain TD96 is not essential and contributes less to heparin binding than the N-terminal HBD. Whether the variant G^{485} in strain TD96 affects its heparin binding activity requires further research.

The N-terminal sequence of CSFV E^{rns} at residues 301 - 311 (PGKICKGVPTH) resembles the potential HBD motif TXXBXXTBXXXTBB (where B designates a basic amino acid, X designates any other residue, and T designates a turn) (Capila and Linhardt, 2002), in which the basic residues K^{303} and K^{306} may provide a positive charge for binding and the last two residues T^{310} and H^{311} may affect the binding activity. Site-directed mutagenesis of both K^{303} and K^{306} to alanine (generating the mutants N227/mKK and N190/mKK) reduced the binding affinity of E^{rns} for heparin (Fig. 3). Further mutation of both T^{310} and H^{311} (generating the mutant N227/mKKTH) had only a slight effect. All the expressed recombinant E^{rns} and mutant proteins retained RNase activities (Fig. 2), confirming that the mutation had no interference with enzyme functional domain. Therefore, the basic residues K^{303} and K^{306} play a critical role in the binding of CSFV E^{rns} to the heparin matrix. The basic residue K^{306} is absent in BVDV E^{rns} probably resulting in malfunction of C-terminal HBD. Furthermore, the results of cell-surface binding assay (Fig. 4) and virus infectivity assay (Fig. 5) also demonstrated that residues K^{303} and K^{306} are important in the binding of the E^{rns} protein to cell-surface GAGs. E^{rns} mutants with K^{303} and K^{306} changed to neutral alanine residues significantly reduced their heparin binding affinities and lost their abilities to inhibit virus infection. The binding ability of E^{rns} to bind to PK-15 cells correlated with its affinity for heparin.

CSFV strain 94.4 (genotype 3) has been epidemic in Taiwan since the 1920s, but the epidemic strain changed to strain TD96 (genotype 2.1) after 1996 (Pan et al., 2005). The residue at position 304 within the N-terminal HBD of TD96 is methionine (M) rather than isoleucine (I), which is conserved in other pestiviruses. Surface-exposed methionine residues can confer antioxidant properties on proteins that may benefit their stability (Levine et al., 1996). The effect of this residue on the HBD-binding activity of E^{rns} and its correlation with viral pathogenesis is currently under studying. In conclusion, a novel potential heparin-binding site in the N-terminal region of CSFV strain TD96 glycoprotein E^{rns}, at residues 301 - 311, has been identified. The two basic amino acids K^{303} and K^{306} within this HBD are crucial for the binding activity of E^{rns} to the heparin matrix and cell-surface GAGs.

Conflict of interest

We declare no conflict of interest.

Acknowledgement

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