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Poly(A)-binding protein interacts with the nucleocapsid protein of porcine reproductive and respiratory syndrome virus and participates in viral replication

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ABSTRACT

Interactions between host factors and the viral protein play important roles in host adaptation and regulation of virus replication. Poly(A)-binding protein (PABP), a host cellular protein that enhances translational efficiency by circularizing mRNAs, was identified by yeast two-hybrid screening as a cellular partner for PRRSV nucleocapsid (N) protein in porcine alveolar macrophages. The specific interaction of PRRSV N protein with PABP was confirmed in infected cells by co-immunoprecipitation and *in vitro* by GST pull-down assay. We showed by confocal microscopy that the PABP co-localized with the PRRSV N protein. Using a series of deletion mutants, the interactive domain of N protein with PABP was mapped to a region of amino acids 52–69. For PABP, C-terminal half, which interestingly interacts other translation regulators, was determined to be the domain interactive with N protein. Short hairpin RNA (shRNA)-mediated silencing of PABP in cells resulted in significantly reduced PRRSV RNA synthesis, viral encoded protein expression and viral titer. Overall, the results presented here point toward an important role for PABP in regulating PRRSV replication.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the most widespread swine diseases in the world and causes enormous economic losses in the swine industry. It mainly causes reproductive failure in sows and respiratory distress in piglets. PRRSV belongs to the family Arteriviridae, which includes equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). Arteriviridae forms the order Nidovirales along with Coronaviridae and Roniviridae families (Cavanagh, 1997; Plana et al., 1992). PRRSV is a single-stranded positive-sense RNA virus. The full-length genenome is about 15 kb nucleotides in size and contains nine open reading frames. ORF1a and ORF1b are located at the 5′ terminal end of the genome and encode a large polyprotein, which can be cleaved into 14 small non-structural proteins (Beerens et al., 2007; Fang and Snijder, 2010; Snijder and Meulenberg, 1998; van Aken et al., 2006). ORF2-ORF7 are located at the 3′ terminal end of the genome and code for major structural proteins (GP5, M and N) and minor structural proteins (GP2, GP3 and GP4) (Jeong et al., 2010; Johnson et al., 2011; Nielsen et al., 2003; Zhou and Yang, 2010).

Nucleocapsid (N) protein is a multifunctional protein. It associates with itself both covalent and noncovalent interactions, providing the basis for viral capsid assembly (Wootton and Yoo, 2003). The nucleocapsid homodimerization occurs on the lumen of the ER and the Golgi complex through disulfide linkages via a cysteine residue at position 23 which is essential for virus replication and infectivity (Wootton and Yoo, 2003). PRRSV replicates exclusively in the cytoplasm of infected cells, however, N protein also accumulates in the nuclei and nucleoli of virus-infected cells (Rowland et al., 1999, 2003). A 'pat-7' motif of PGKKNKK at positions 41–47 aa has been identified as the functional nuclear localization signal (NLS) (Rowland et al., 2003). N protein nuclear localization is associated with virulence of PRRSV since modification in the NLS causes attenuation of the virus (Lee et al., 2006a,b; Pei et al., 2008) suggesting that N protein localization in the nucleus may play a role in viral pathogenesis.

The precise mechanism of N protein trafficking within a cell and to the nucleolus is unknown, although N protein has been shown to interact with the small nucleolar RNA (snoRNA)-associated protein fibrillarin and thus may potentially localize to the nucleolus via this interaction (Yoo et al., 2003). Another host cell protein, the inhibitor of MyoD family-'a' (I-mfa) domain-containing protein (HIC), was identified as a molecular partner with N (Song et al., 2009). The fact that N protein localizes in the nucleolus and interacts with nucleolus proteins makes us concerning N may has functions in recruiting host cell proteins to facilitate virus replication. In the present study, we sought to identify new cellular proteins interacting with PRRSV N. Knockdown of N interactive host cell protein expression may be helpful to reduce viral replication.

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2. Materials and methods

2.1. Cells and viruses

Marc-145 cells (Provided by Dr. M Tan in Iowa State University) were grown as monolayer in Dulbecco's modified Eagle's medium (Gibco) supplemented with 8% fetal bovine serum (FBS, heat-inactivated), 100 IU of penicillin g/ml and 100 μl of streptomycinsulfate/ml, and cultured at 37 °C with 5% CO_2. The North American genotype PRRSV strain SY0608 (isolated and kept in our lab) was used throughout the study.

2.2. Antibodies

The polyclonal rabbit antiserum specific to PABP was purchased from Abcam. The monoclonal antibody anti-GADPH was purchased from Abmart. The monoclonal antibody anti-histidine tag was purchased from Abmart. The secondary antibodies goat anti mouse and goat anti rabbit labeled with horseradish peroxidase (HRP) were purchased from Boster. The secondary antibodies goat anti mouse and goat anti rabbit labeled with Alexa 488 and Alexa 555 respectively were purchased from Beyotime. Monoclonal antibodies to PRRSV N protein and swine serum anti-PRRSV were made in our lab.

2.3. cDNA library construction and yeast two-hybrid screening

Total RNA was purified from the porcine alveolar macrophages (Landrace × local stock) by using TRIzol Reagent (Invitrogen) and 1 μg was used to synthesize first-strand cDNA using Smart oligo (dT) (Clontech) as primer. A cDNA library was prepared using the "Make Your Own 'mate & plate' Library System kit" (Clontech), by 33 cycles of PCR amplification with specific primers supplied by the vendor. The amplified cDNA was transformed in *Saccharomyces cerevisiae* strain Y187 together with predigested $pGADT_7$ vector containing a leucine-selectable marker. Transformants were plate amplified on synthetic defined drop-out medium lacking leucine (SD/-Leu), aliquoted, and stored as a pretransformed yeast library. The full-length PRRSV ORF7 was cloned by PCR using ORF7 cDNA template and ligated into $pGBKT_7$ with *EcoR* I and *Sal* I (Takara). The $pGBKT_7$-ORF7 plasmid was transformed into *S. cerevisiae* strain Y2H and mated with the pretransformed yeast Y187 cDNA library. Progeny were plated on SD/-Leu/-Trp medium containing 40 μg/ml of X-α-gal (5-bromo-4-chloro-3-indolyl-α-galactopyranoside, Clontech) as chromogenic substrate and 125 ng/ml Aureobasidin A results in considerably lower background. Growing colonies were replicated on SD/-Ade/-His/-Leu/-Trp medium containing X-α-gal and Aureobasidin A, positive yeast diploids were identified as blue colonies. Colonies were picked and individual plasmids were amplified, purified, and analyzed by restriction endonuclease reaction. Automated dideoxy DNA sequencing followed by analysis using the NCBI Blast search program revealed the identity of insert DNA in positive clones.

2.4. Immunoprecipitation assay

Marc-145 cells were infected with the SY0608 strain of high pathogenic PRRSV. Thirty-six hours post infection, cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA) containing complete protease inhibitors (Sigma) on ice for 5 min. Lysates were cleared by centrifugation (10,000*g*), and the supernatants were incubated with PABP/N antibody overnight at 4 °C with rotation, then 50% suspension protein G-Sepharose was added and incubated for 3 h at 4 °C. Beads containing protein complexes were washed by 5 times with lysis buffer after which the complexes were eluted by boiling for 5 min in 2× gel sample buffer. Proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The PABP or N protein was detected by immunoblotting as following.

2.5. Immunoblotting

Proteins were fractionated by SDS-PAGE and then blotted onto nitrocellulose membranes (Pall). Membranes were incubated with the primary antibodies rabbit anti-PABP (Abcam) at a 1:500 dilution; or mouse anti-N protein IgG, 1:500; mouse anti-GAPDH (Abmart), 1:2000. After incubation with horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (Boster), the immunoreactive proteins were detected using ECL chemiluminescent chromogenic kit (Biouniquer) and exposure to X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.6. Indirect immunofluorescence microscopy

Marc-145 cells cultured on glass coverslips were infected with PRRSV (strain SY0608) at 10 MOI. Cells were processed for indirect immunofluorescence 48 h post-infection (hpi) by fixing in 4% paraformaldehyde for 30 min. Cell membranes were permeabilized by incubating with PBS buffer containing 0.2% Triton X-100 for 5 min. Samples were blocked in 1%BSA for 30 min before incubation with primary and secondary antibodies. All the washes were done in PBS buffer. PRRSV N protein was detected with mouse mAb anti-N protein at a 1:200 dilution. PABP was detected with rabbit polyclonal antibody anti-PABP (Abcam) at a 1:500 dilution. The secondary antibodies utilized in these studies were Alexa Fluor 555-conjugated goat anti-rabbit (Beyotime) and Alexa Fluor 488-conjugated goat anti-mouse (Beyotime). Coverslips were mounted onto microscope slides and samples were examined using a Zeiss LSM700 confocal microscope.

2.7. Plasmid constructions

The N gene sequence of SY0608 (GenBank ID: EU144079.1) was amplified from a plasmid containing PRRSV strain SY0608 cDNA using a pair of primers (forward 5′-GCC*GAATTC*ATGCCAAATAACAA CGGCAAGCAG-3′ and reverse 5′-GAC*GGATCC*TCATGCTGAGGGTGA-TGCTGTGGC-3′) where inclined form letters indicate *EcoR* I and *Sal* I recognition sequences, respectively and cloned into the *EcoR* I and *Sal* I sites of $pGBKT_7$ (Clontech) to construct bait plasmid $pGBKT_7$-ORF7. The N gene was digested from $pGBKT_7$-ORF7 with *EcoR* I and *Sal* I, then cloned into pET32a expression vector system (Invitrogen). The ORF7 derivative mutants, the carboxy-terminal deletion mutants (C11, C50, C66, C86 and C98) and the amino-terminal deletion mutants (N18, N30, N52, and N69) were constructed by using pET32a expression vector system. The primers used to amplify the N derivative deletion mutants were listed as follow Table 1. The PABP coding sequence (GeneBank ID: XM_001927747.1) was amplified from the Marc-145 cell cDNA clone using a pair of primers (forward 5′-TAT*GAATTC*ATGAACCCCAGCGCCCC-3′ and reverse 5′-GCG*CTCGAG*TTAAACAGTTGGAACAC-3′), where inclined form letters indicated *EcoR* I and *Xho* I recognition sequences, respectively, and cloned into the *EcoR* I and *Xho* I sites of pGEX-6p-1 (Invitrogen). Expression plasmids encoding the N- and C-terminal regions of PABP were constructed as follows. The coding region for the N-terminal region of PABP (amino acid residues 1–368, was amplified by PCR using a pair of primers (forward 5′-ATA*GAATTC*ATGAACCCCAGCGCCCC-3′ and reverse 5′-CTG*CTC-GAG*TCAAGCTAAAGCTACA-3′) where inclined form letters indicate *EcoR* I and *Xho* I site. The coding region for the C-terminal region of PABP (amino acid residues 369–636) was amplified by PCR using

Table 1
Primers used for PCR amplification for ORF7 derivative mutant genes.

Primers name	Sequence (direction 5′–3′)
C11 F	GGC*GAATT*CATGCCAAATAACAACGGC
C11 R	ATA*GTCGAC*TCACACAGTATGTTGCGTC
C50 F	GGC*GAATT*CATGCCAAATAACAACGGC
C50 R	ATA*GTCGAC*TCATTGCCGCTCACTAG
C66 F	GGC*GAATT*CATGCCAAATAACAACGGC
C66 R	CAG*GTCGAC*TCATAGAGGGAAATGGGG
C86 F	GGC*GAATT*CATGCCAAATAACAACGGC
C86 R	GCG*GTCGAC*TCATCTGGACTGGTTTTGTT
C98 F	GGC*GAATT*CATGCCAAATAACAACGGC
C98 R	TAG*GTCGAC*TCACATTTGGCACAGCTGATT
N18 F	GAC*GAATT*CATGCCAGTCAATCAGCTGTGCCA
N18 R	ATA*GTCGAC*TCATGCTGAGGGTGATGCT
N30 F	ATA*GAATT*CATGATCGCCCAACAAAACCAGT
N30 R	ATA*GTCGAC*TCATGCTGAGGGTGATGCT
N52 F	ATA*GAATT*CATGAAGCCCCATTTCCCTCTAG
N52 F	ATA*GTCGAC*TCATGCTGAGGGTGATGCT
N69 F	CTG*GAATT*CATGCCTAGTGAGCGGCAATTGT
N69 R	ATA*GTCGAC*TCATGCTGAGGGTGATGCT

The inclined form letters indicate *EcoR* I and *Sal* I recognition sequences.

a pair of primers (forward 5′-GCG*GAATTC*ATGCAGCGCAAAGAAGA-3′ and reverse 5′-CTG*CTCGAG*TCATTAAACAGTTGGA-3′) where inclined form letters indicate *EcoR* I and *Xho* I site. The *Escherichia coli* strains BL-21 and DH5α were used as hosts for generating mutant genes and for general purpose cloning, respectively.

2.8. Protein expression in E. coli and pull-down assay

The N protein and deletion mutants were expressed in *E. coli* as a Histidine-tagged fusion protein. PABP deletion mutants were expressed in *E. coli* as GST fusion protein. 5 ml of Luria-Bertani medium containing 100 μg of ampicillin/ml was inoculated with 1/100 of an overnight culture and grown to an optical density of 0.6 at 600 nm. Protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The bacteria were collected at 12,000*g* for 1 min, and the pellet was resuspended in 500 μl of PBS and sonicated on ice three times for 5 s each time with 5 s intervals (model W-385; Ultrasonics Inc.).

The insoluble fraction and cell debris were removed by centrifugation at 12,000*g* for 1 min at 4 °C. The supernatants were incubated with 20 μl of 50% slurry of Ni-NTA Agarose beads (invitrogen) for 3 h at 4 °C with constant agitation. The beads bound to the histidine-tagged fusion proteins were collected at 1,000*g*, washed 5 times in PBS, then incubated with Marc-145 cell lysates overnight at 4 °C with rotation. Beads containing protein complexes were washed 5 times with PBS after which the complexes were eluted by boiling for 5 min in 2× gel sample buffer. Proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.9. Design and cloning of plasmids expressing short hairpin RNA

The target sequences of PABP-specific small interfering RNA (siRNA) were selected by using Ambion online. Oligonucleotides were synthesized and constructed into the *Kpn* I and *Bgl* II sites of pSUPER vetctor (Invitrogen). The oligonucleotides sequences were listed in Table 2. The expressed hairpins that produce 21 nt siRNA with 19 bases (inclined form letters) of homology to PABP. Meanwhile, another sequence in a random order as PABP#1 was designed, and recombinant pSUPER-PABP-1rd# was constructed and used as a negative control.

2.10. Gene silenced mediated by short hairpin RNA (shRNA)

Marc-145 cells were seeded in 24-well plates with 2×10^4cells/well in DMEM 24 h before transfection. Marc-145 cells were transfected with 0.8 μg plasmid expressing shRNA using a TransFast™ Transfection Reagent (Promega) follow the manufacturer's instructions. Efficiency of PABP expression knockdown was detected by Western blotting with antibody to PABP (Abcam) as above Immunoblotting. To study the effect of PABP knockdown on viral RNA synthesis in a single replication cycle, Marc-145 cells were transfected with PABP-specific shRNA or nonspecific shRNA. After incubation for 24 h, cells were washed with Hank's and infected with SY0608 virus strain at 10 MOI. After incubation at 37 °C, for different time, the viral samples were harvested and detected with Quantitative RT-PCR assays, Immunoblotting and $TCID_{50}$.

Table 2
The design of siRNA targeting to PABP gene.

Designation	Sequence (direction 5′–3′)	Genome position in PABP
pSUPER-PABP 1#	AAGGTGGTTTGTGATGAAAAT	387–405
pSUPER-PABP 2#	AACTAAGACCAAGTCCTCGCT	1291–1309
pSUPER-PABP 3#	AAATGTTGGGTGAACGGCTCT	1681–1699
pSUPER-PABP 1rd#	AAGTATGAATTATAAGTGGGT	

2.11. Quantitative RT-PCR assays

The viral RNA was extracted from the cellular samples using TRIzol reagent (Invitrogen). The cDNA was reverse-transcribed from 1 μg of total RNA using oligo (dT) primers. Quantitative RT-PCR was carried out by using SYBR green master mix (Toyob) and specific primer sets for detecting the mRNA levels of PRRSV N protein gene. The primers were designed by using the Primer3 as following: forward 5′-AATAACAACGGCAAGCAGCAA-3′ and reverse 5′- CCTCTGGACTGGTTTTGTTGG-3′. Meanwhile, a pair of primers (forward 5′-CTCCATCATGAAGTGCGACGT-3′ and reverse 5′-GTGATCTCCTTCTGCATCCTGTC-3′) were used for amplify the β-actin gene which used as an internal control. A reaction mix of 20 μl was composed of 10 pmol of each gene-specific primer, 10 μl of SYBR green master mix, and 1 μl of cDNA. Amplification reactions were performed under the following conditions: 2 min at 95 °C, 40 cycles for 15 s at 95 °C, and 1 min at 60 °C. Relative transcript levels were calculated using ΔΔCt method as specified by the manufacture. The specificity of the assay was confirmed by melting-curve analysis at the end of the amplification program (95 °C 15 s, 60 °C 1 min, 95 °C 15 s).

2.12. Statistical analysis

All data were presented as mean ± S.D. All statistical evaluations were conducted by use of the SPSS statistical software, Version 16.0 (SPSS Inc., Chicago, IL). The viral mRNA and virus titers data were evaluated by One-way repeated measurement ANOVA and Least significance difference (LSD). The differences between different groups were determined by student's t-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Yeast-two hybrid screening of N binding proteins

To define novel cellular proteins that interact with PRRSV N protein, a yeast two-hybrid screening assay was employed. The full-length PRRSV ORF7 cDNA was cloned into a bait plasmid $pGBKT_7$. After screening as the instruction of "Matchmaker™ Gold Yeast Two-Hybrid System User Manual" (Clontech), 5 blue clones were detected from the PAM cDNA library. And the validity of these clones was confirmed by plating onto synthetic selection media QDO/-Leu/-Trp/-Ade/-His/X-α-gal/Aureobasidin (Fig. 1A).

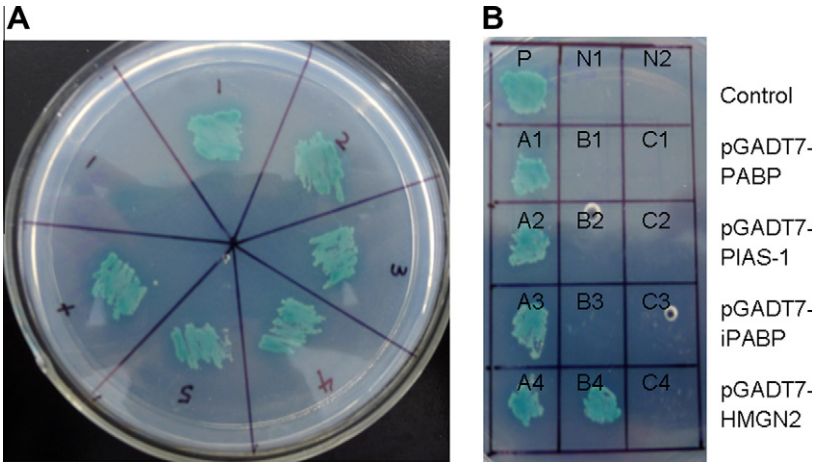

Fig. 1. The blue clones screened from yeast two-hybrid screen assay (A) and the individual plasmids DNA identified by PCR and restriction endonuclease reaction (B) Five blue clones screened from yeast two-hybrid screen assay were picked respectively and the validity was tested by plating onto synthetic selection media QDO/-Leu/-Trp/-Ade/-His/X-α-gal/Aureobasidn. Yeast cells contain both pGADT$_7$-T and pGBKT$_7$-p53 or pGBKT$_7$-Lam used as a positive and negative control. (B) PABP, PIAS-1, iPABP and HMGN2 derived prey plasmid were reintroduced into fresh Y187 with pGBKT$_7$-N bait plasmid. Positive for interactions showed blue. P, transformed pGADT$_7$-T and pGBKT$_7$-p53 as a positive control; N1, co-transformed pGADT$_7$-T and pGBKT$_7$ as a negative control; N2, transformed pGADT$_7$-T as negative control; A1-4, transformed pGBKT$_7$-ORF7 and the individual prey plasmid; B1-4, transformed pGBKT$_7$ and the individual prey plasmid; C1-4, transformed the individual prey plasmid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Cellular proteins positive for interaction with PRRSV N protein through Yeast two hybrid and NCBI sequence analysis.

No.	Protein name	Accession	Description	Query coverage (%)	*E* value	Max ident (%)
1	PABP	XM_001927747.1	Sus scrofa similar to Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1) (LOC100153958), mRNA	75	0	99
2	PIAS-1	NM_001075396.1	Bos taurus protein inhibitor of activated STAT, 1 (PIAS1), mRNA > gb|BC114146.1|	94	0	95
3	iPABP	NM_001083724.1	Bos taurus poly(A)-binding protein, cytoplasmic 4 (inducible form) (PABPC4), mRNA > gb|BC134509.1|	77	0	95

In order to identify the gene responsible for the positive interaction, the individual plasmid DNA of these 5 blue yeast clones grown on the synthetic selection media were extracted and analyzed by restriction endonuclease reaction. DNA sequencing followed by analysis using the NCBI Blast search program revealed the insert DNA in positive clones were as follows: PABP (poly(A)-binding protein 1, GeneBank ID: XM_001927747.1), HMGN-2 (high-mobility group nucleosomal binding domain 2, GeneBank ID: NM_001098945.1), PIAS-1 (protein inhibitor of activated STAT 1, GeneBank ID: NM_001075396.1, iPABP (inducible poly(A)-binding protein, GeneBank ID: NM_001083724.1).

To confirm the screening clones, pairwise yeast two-hybrid assays were used. Prey plasmids were isolated from Ade+, His+, X-α-galactosidase+, Aureobasidin A+ yeast clones. The prey plasmids were reintroduced into fresh Y187 yeast together with bait plasmid pGBKT$_7$-N. As shown in Fig. 1B, PABP1, PIAS-1, iPABP, and HMGN-2-derived preys tested were positive (blue) for interaction with PRRSV N bait. When co-transformed these prey plasmids with pGBKT$_7$, PABP1, PIAS-1, iPABP-derived preys were negative. But HMGN-2-derived prey tested was positive (showed blue as in Fig. 1B. B4). These data indicated that the interactions between PABP, PIAS-1, iPABP and N were genuine positive, but the interaction of HMGN-2 and N was false positive. In a word, 3 cellular proteins were obtained as the candidates for the interaction of PRRSV N by yeast two hybrid screening assay, as shown in Table 3.

3.2. PABP-N interaction was confirmed in vitro and in vivo

To confirm the interaction between N and PABP, we first designed a GST-pulldown assay to verify that PABP and N protein both expressed in *E. coli* can bind with each other. PABP was expressed in *E. coli* as the GST-PABP fusion protein and coupled to glutathione-Sepharose beads. N protein was expressed in *E. coli* as the Histhione-tagged-N fusion protein and incubated with glutathione-Sepharose beads bound to either GST or the GST-PABP. As shown in Fig. 2A, N protein was detected as protein bound to GST-PABP coupled beads.

To examine if PABP interacts with PRRSV N in the context of PRRSV infection, we immunoprecipitated virus-infected Marc-145 cell lysates with anti-PABP antibody and probed for presence of N by using an N antibody which detects N protein. The results showed that N protein could form stable complexes with PABP in PRRSV-infected Marc-145 cells (Fig. 2B).

To determine whether the N-PABP interaction was sensitive to nuclease treatment, the samples were treated with RNase A at 37 °C for 30 min before adding antibodies, and then co-immunoprecipitation and immunoblotting were accomplished as above described. The result showed that RNase A treatment did not significantly alter the interaction of PABP and N (Fig. 2C). These results indicated that the PABP-N interaction is not mediated by the intact RNA.

3.3. PABP colocalized with PRRSV N protein

To further investigate the interaction of N protein with PABP, Marc-145 cells were infected with SY0608 strain of PRRSV or mock-infected and then processed for indirect immunofluorescence microscopy. Colocalization of proteins, as revealed by yellow immunostaining of merged images, was evident only when cells expressed combinations of the PABP and N protein (Fig. 3). Here

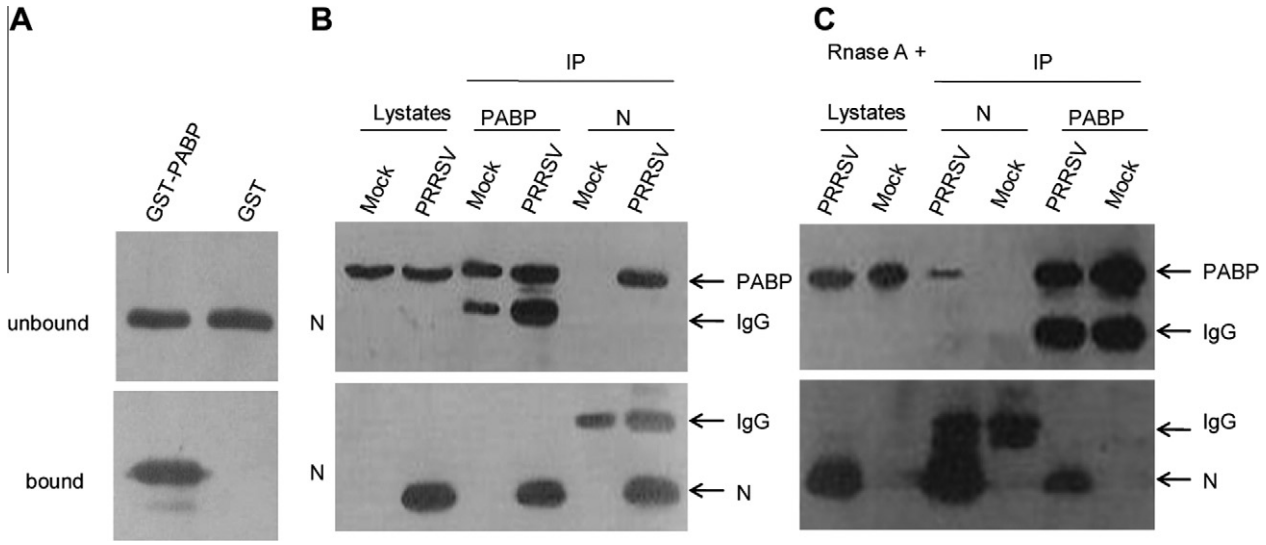

Fig. 2. PABP-N interaction was examined by immunoprecipitated assay. (A) The N protein could interact with PABP *in vitro*. PABP was expressed in *E. coli* by plasmid pGEX-6p-1-PABP as the GST-PABP fusion protein and coupled to glutathione-Sepharose beads. Then they were incubated by PRRSV infected Marc-145 cell lysates. N protein was detected by western blot with anti-N monoclonal antibody. (B) N protein interacted with PABP in the virus-infected cells. Marc-145 cells were infected with PRRSV, and lysates were prepared at 36 hpi. Lysates were immunoprecipitated (IP) with monoclonal antibodies against N or PABP and then subjected to western blot analyses with antibodies to N and PABP. (C) RNA is not required for the interaction between PABP and N. Samples were treated with RNase A for 30 min at 37 °C, Lysates were immunoprecipitated with monoclonal antibodies against N or PABP and then subjected to western blot analyses with antibodies to N and PABP.

we see, PABP distributed evenly in cytoplasm in mock infected cells but its appearance was like granule both in nucleus and cytoplasm in PRRSV infected cells.

3.4. Identification of interactive domain on PRRSV N protein and PABP

To define the primary sequence required for N-PABP interactions, a series of Histidine-tagged-N fusion constructs were generated by progressively deleting amino acids from either the amino or carboxy terminus of the N protein. The each Histidione-tagged fusion fragment protein was expressed in *E. coli* (Fig. 4A), and was individually coupled to Ni-NTA Agarose beads (Invitrogen). PABP supplied by cell lysates was added to the Agarose beads to determine the interactions between N and PABP by the His pull-down assay. PABP and Histidione-tagged fusion N proteins were identified by using anti-PABP antibody (Fig. 4B) and anti-His-tag antibody (Fig. 4C) and N specific porcine serum (data not shown). The results showed that the C terminus N protein mutants (C11, C50 and C66) and the N terminus mutants (N18 N30, and N52) could bind to PABP. But the mutants (C86, C98, and N69) which lacked the 52-57 amino acid regions did not interact with PABP. However, the two key deletion mutants (C86 and C98) express low levels of protein (Fig. 4B). Therefore, absence of a PABP band could simply be due to an amount of protein below the detection limit of their western blotting. Mutant N69 shows good expression, so the binding region would be more reasonable to state in aa 52–69.

3.5. N protein binds to the C terminus of PABP

PABP is a multifunctional protein composed of multiple domains. To determine which region of PABP is required for binding to N protein, the N- and C-terminal regions of PABP, PABP-NT and PABP-CT (Fig. 5A), were expressed as GST fusion proteins in bacteria and then immobilized on glutathione agarose beads (Fig. 5B). The beads were incubated with PRRSV infected Marc-145 cell lysates. After washing, the bound proteins were eluted and detected by immunoblotting with antibodies to N protein. As shown in Fig. 5C, N protein binds to the C terminus of PABP but not the N terminus nor GST alone.

3.6. Decreasing expression PABP mediated by shRNA reduces PRRSV replication

The observations that PABP interacts with the PRRSV N protein led us to hypothesize that the interaction plays important roles in the viral life cycle. To analyze the role of PABP in PRRSV replication, RNA interference was used to knock down PABP mRNA. As shown in Table 2, 4 plasmids were constructed and individually transfected into Marc-145 cells. The results showed PABP expression in the cells transfected with pSUPER-PABP1# was reduced remarkably compared with those in other two candidates and control groups (Fig. 6 A).

To discover whether PABP knockdown had any effect on PRRSV virus replication, the viral mRNA of ORF7 genes were examined in PRRSV-infected Marc-145 cells, with or without pSUPER-PABP1#, by using quantitative RT-PCR. As shown in Fig. 6B, viral mRNA levels were decreased in pSUPER-shPABP1# treated group by approximately 40 percent compared with those in control or mock-transfected groups. Meanwhile, the viral N protein expression levels were decreased obviously in the pSUPER-shPABP1# group by detecting with western blotting (Fig. 6C). By contrast, the levels of GAPDH were not affected.

Furthermore, the virus yield in the culture supernatants was significantly lower than those in the nonspecific shRNA or no shRNA treatment groups ($p < 0.05$) (Fig. 6D). In summary, the results demonstrate that short hairpin RNA (shRNA)-mediated silencing of PABP efficiently reduced the PRRSV load in cell culture with regard to viral RNA synthesis, viral encoded protein expression and viral titer.

4. Discussion

In the present study, we have identified a host cell encoded N-binding protein, PABP. This protein generally plays a critical role in regulating initiation of translation and is one of the central targets

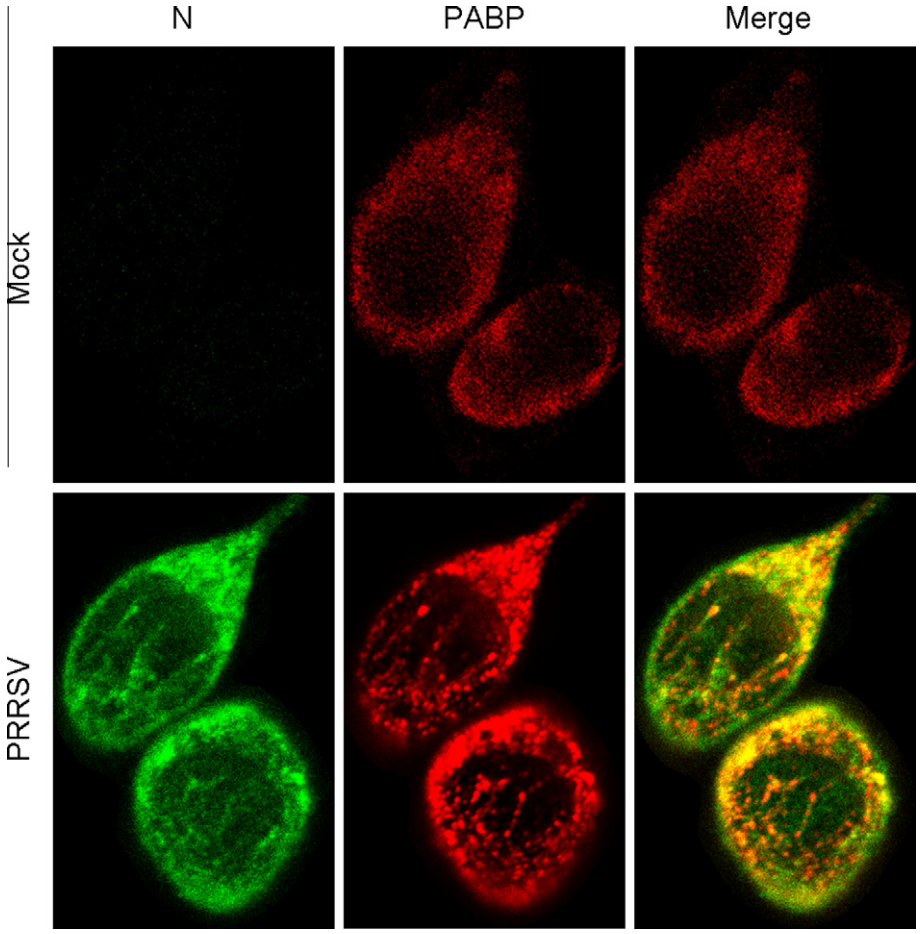

Fig. 3. Interaction of PABP with N protein in PRRSV-infected Marc-145 cells. PABP colocalizes with PRRSV N protein in the cells. After incubation for 48 h, the PRRSV-infected and mock infected cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton-X100. The N protein and PABP were detected with mouse and rabbit antibodies, respectively. Primary antibodies were detected with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555. The cells were examined by laser scanning confocal microscopy. PRRSV-infected cell costained with N-specific mouse mAb (green) and PABP-specific rabbit polyclonal antibody (red). The yellow regions are the areas where N protein and PABP colocalized. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

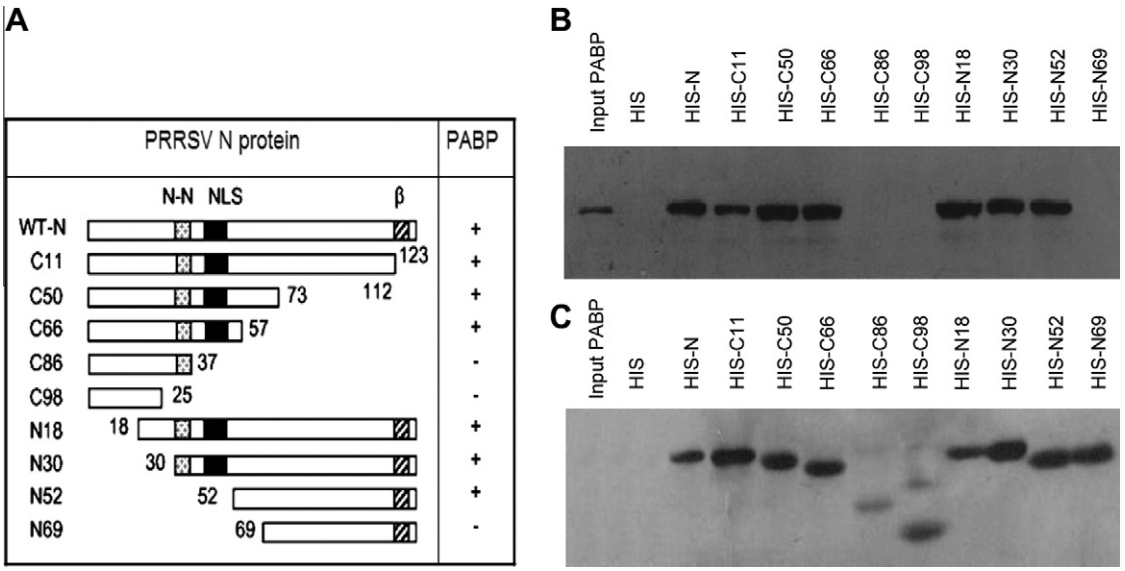

Fig. 4. Identification of the domain of N protein interacted with PABP. The N gene was progressively deleted from either the 5′ or the 3′ terminus, and the fragments were individually expressed in *E. coli* as Histidine-tagged fusion proteins. The expressed N mutant proteins were coupled to Ni-NTA Agarose beads, and the beads were complexed with cell lysates prepared from Marc-145 cells. The beads were washed by five times, and the bound protein was analyzed by SDS-PAGE and Western blot with anti-PABP antibody (B) and anti-His-tag antibody (C). WT, wild-type; N–N, N protein-N protein interactive domain; NLS, nuclear localization signal; β, β-strand conserved among arteriviruses; + and − indicate binding affinity between N and PABP; the numbers indicate amino acid positions.

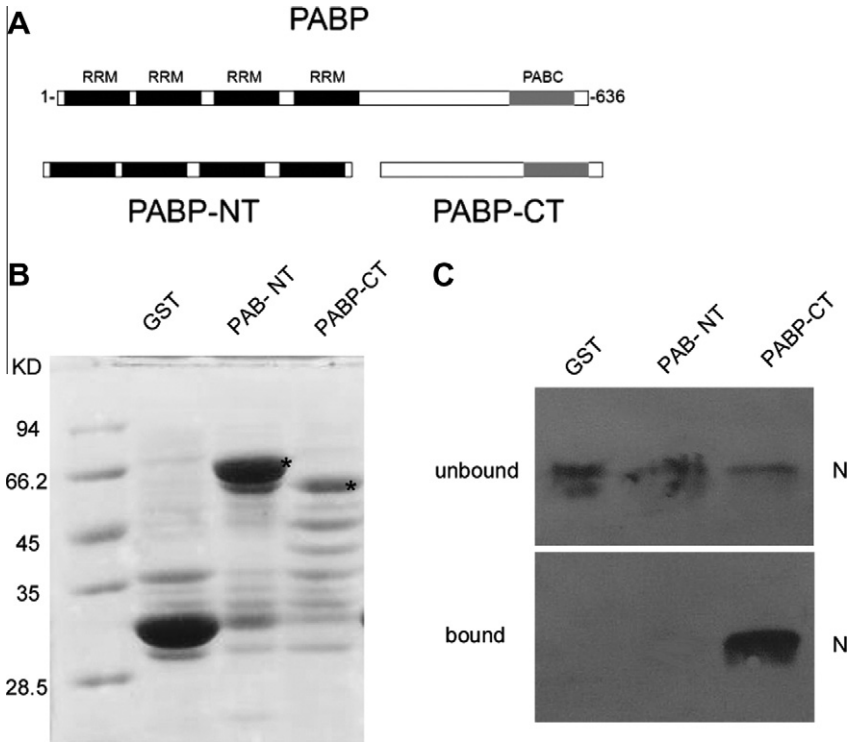

Fig. 5. Identification of the domain of PABP interacted with N protein. (A) Schematic of human PABP. The N terminus (NT) contains four tandem RNA recognition motifs (RRMs), and the C terminus (CT) contains a PABC domain that mediates protein-protein interactions with regulatory proteins. (B) GST-tagged NT and CT fusion proteins (indicated by a star) were expressed in *E. coli and* separated by SDS-PAGE and then stained with Coomassie blue. (C) Purified Histidine-tagged N expressed in *E. coli* was incubated with the glutathione Sepharose pre-bound with PABP NT, PABP CT, or GST alone. The elutes were subjected to SDS-PAGE and immunoblotting with anti N protein antibodies.

of viruses that cause shutdown of host cell protein synthesis (Smith and Gray, 2010). It has been well established for years that many RNA and DNA viruses partially or completely inhibit host cell translation as a mean to interfere with cellular defense systems (Kuyumcu-Martinez et al., 2002; Lee and Glaunsinger, 2009). Data also shows that PABP has a direct function in regulating viral RNA synthesis. PABP bound to the picornavirus (PV) poly(A) tail appears to play a direct additional role in viral negative-strand synthesis (Herold and Andino, 2001). Gene silencing of host cell PABP causes a significant 2 fold reduction of TGEV viral RNA synthesis (Galan et al., 2009).

It would not be surprising if PRRSV has a similar requirement for PABP. Our data suggested PABP specifically bound with the N protein and its silencing caused an approximately 50% reduction in viral RNA level that correlated with a detectable reduction in virus titers. It indicates that PABP in general plays a role in PRRSV replication. The biological effects of preventing the PABP-N interaction did not appear to be severe, suggests the inhibition of PABP-PRRSV-N interaction may not be essential for inhibiting viral replication, and it indicates that a more thorough understanding of the interaction is needed. According to Beura et al., poly(A)-binding protein cytoplasmic 4 (PABP4), which is isolated as an activation-induced T-cell mRNA encoding a protein, was associated with PRRSV nonstructural protein 1β (nsp1β) (Beura et al., 2011). Numerous cellular proteins (PABP and inducible PABP were also included) seemed to be involved in interactions with PRRSV N, functions of these proteins involved translation, mRNA stability, splicing, RNA binding and so on (Jourdan et al., 2012a). In the future, we will investigate the affinity of the PABP-PRRSV-N interaction and it will be interesting to explore the possible involvement of the other interactions in PRRSV life cycle. The N protein binds viral RNA for encapsidation (Jourdan et al., 2012b). The nidovirus genomes contain a 5′ cap structure, 5′ and 3′ NTRs, and a poly(A) tail. Arteriviridae genomes mimic cellular mRNAs and are presumed to translate their genomes in a cap and poly(A)-dependent manner. The binding of cap-recognizing factors to the 5′ end and the interaction of the PABP with the N protein could circularize the viral genome for translation. This mechanism would favor the recycling of ribosome, the selection of complete molecules as templates and the controlled regulation of the switch between translation and RNA synthesis that occurs in opposite directions. With our data, *in vitro* binding assays using purified protein fragments revealed that the N protein binds to the C terminus of PABP. This region of PABP contains a PABC domain that interacts with PAM2 motifs (Albrecht and Lengauer, 2004; Kozlov et al., 2004) which are found in positive (Paip1) and negative (Paip2) regulators of translation (Khaleghpour et al., 2001a,b; Kozlov et al., 2001; Roy et al., 2002). When the N protein levels are high, it may compete with positive regulators of translation, such as Paip1, for binding to PABP. The Paip1-PABP complex is thought to function in ribosome recruitment and translation initiation through interactions with eIF4A and other translation factors (Roy et al., 2002).

Our results suggested the interactive domain of N protein with PABP was mapped to a region of amino acids 52–69. It is also noted that a recent report mapped the PABP binding site(s) by employing a series of N-protein mutants containing sequential alanine substitutions expressed in cells as YFP-fusion proteins and suggested that PABP did not interact with N in case of amino acid 61–70, 81–90, 91–100 and 101–110 were respectively replaced by alanine (Jourdan et al., 2012a). However, it is known that N protein showed monomers, dimers, trimers and tetramers under native conditions and the oligomeric state and valency of molecules can influence binding affinity (Jourdan et al., 2012b). The report also showed alanine blocks within the well-structured C-terminal half (CTH) had a

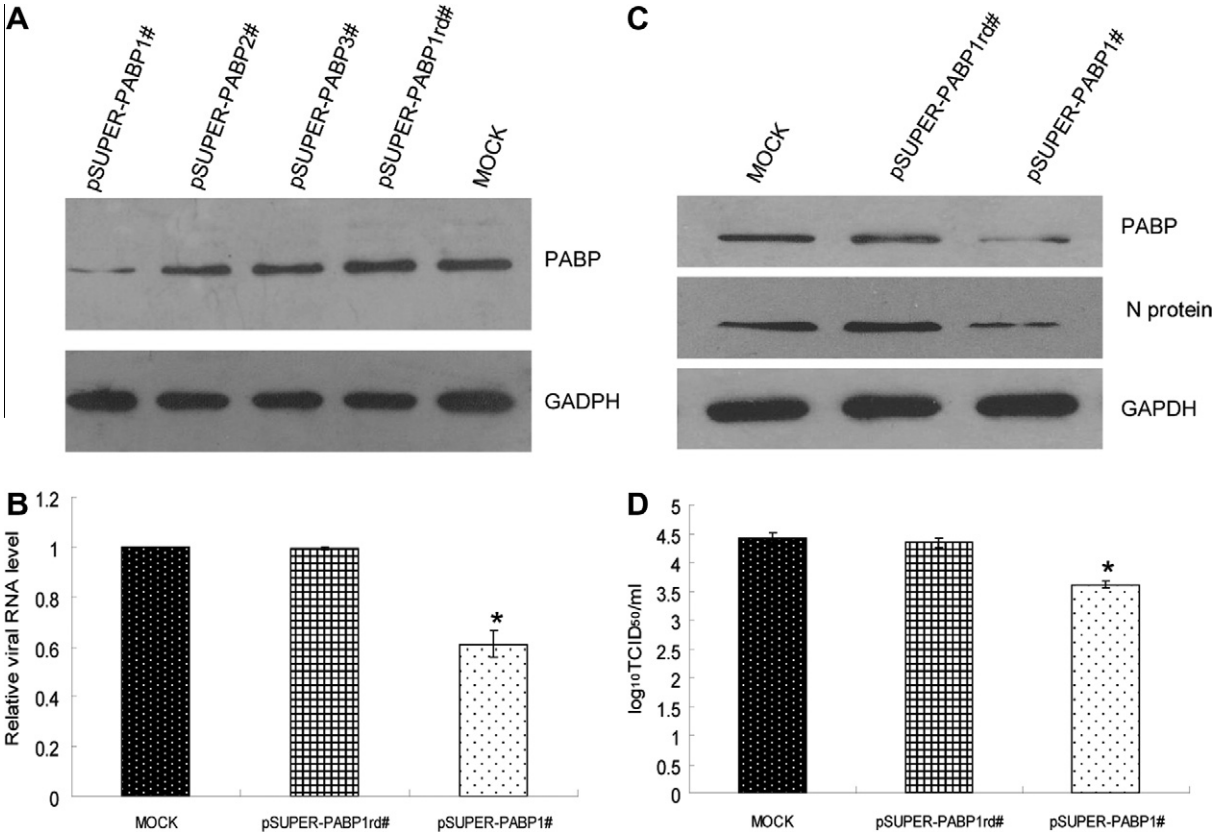

Fig. 6. Knockdown of PABP by shRNAs reduces PRRSV replication. (A) Screened specific shRNA to PABP. pSUPER-PABP1#, pSUPER-PABP2#, pSUPER-PABP3#, and pSUPER-PABP1rd# negative control plasmids DNA were individually transfected into Marc-145 cells. At 48 hpi, the cells were lysised, and PABP and GAPDH were detected by western blotting. (B) Effect of shRNA on PRRSV RNA replication. Marc-145 cells were transfected with pSUPER-PABP1# and with pSUPER-PABP1rd# control plasmid. After incubation for 24 h, the cells were infected with PRRSV at 1 MOI. At 12 hpi, total cellular RNA was extracted and the mRNA levels of PRRSV N gene were determined by quantitative RT-PCR. (C) Effect of shRNA on PRRSV viral protein expression. Marc-145 cells were transfected and infected as before. At 24 hpi, the cells were lysised, and three proteins (PABP, N protein and GAPDH) were detected by Western blotting. (D) Effect of shRNA on PRRSV titers. In another test, Marc-145 cells were also transfected and infected as before, and cell cultures were harvested at 48 hpi and PRRSV titers were detected as $TCID_{50}$.

more dramatic effect on oligomer formation of N protein: Mutants N61–70, N81–90, N91–100 and N101–110 were mainly present as monomers (Jourdan et al., 2012b). Therefore, low affinity of PABP and mutants N61–70, N81–90, N91–100 and N101–110 could be due to oligomeric state of N mutants.

PABP is a nucleo-cytoplasmic shuttling protein, which in the absence of extra physiological perturbations, shows almost exclusive cytoplasm steady-state localization (Afonina et al., 1998). Nuclear redistribution of PABP has been reported to occur during infection with a number of unrelated viruses (Harb et al., 2008; Ilkow et al., 2008; Montero et al., 2008). Regarding to our study, PABP was seen evenly distributed mainly in cytoplasm in mock-infected, however, it seemed to be granule appearance to gather both in nucleus and cytoplasm in PRRSV infected Marc-145 cells. On the basis of N-PABP interaction, it is logical to propose that PRRSV N recruits PABP accumulates at sites of viral synthesis and/or sequesters it to inhibit cellular protein synthesis. The effect of N protein on the physiology of host cells remains to be investigated.

These studies of the shRNA target PABP not only improve our understanding of the replication of PRRSV, but also have the potential for use as the basis for developing a drug against PRRSV that acts by inhibiting PRRSV replication. If the interaction between PABP and PRRSV-N could be proved to be important for the virus replication and virus virulence by using infectious cDNA techniques in the future, the specific mutation of N protein might result in attenuated viruses and which could make the mutation PRRSV an interesting candidate for vaccine development. In addition, investigations into the cellular protein involved in the proliferation of PRRSV will improve our understanding of the molecular basis of pathological phenomena of virus infection.

Acknowledgements

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