Virus Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

The interaction between host Annexin A2 and viral Nsp9 is beneficial for replication of porcine reproductive and respiratory syndrome virus

Jiangnan Li[a,1], Dongwei Guo[b,1], Li Huang[a], Manman Yin[a], Qingfang Liu[a], Yan Wang[a], Chunmei Yang[a], Yuanyuan Liu[a], Lijie Zhang[a], Zhijun Tian[a], Xuehui Cai[a], Liyun Yu[b], Changjiang Weng[a,*]

[a] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, Heilongjiang, China
[b] College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, Heilongjiang, China

ARTICLE INFO

Article history:
Received 29 January 2014
Received in revised form 6 May 2014
Accepted 7 May 2014
Available online xxx

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Non-structural protein 9 (Nsp9)
Annexin A2 (ANXA2)
Yeast two-hybrid (Y2H)
Viral replication

ABSTRACT

Non-structural protein 9 (Nsp9), a RNA-dependent RNA polymerase (RdRp) of the porcine reproductive and respiratory syndrome virus (PRRSV), is necessary for PRRSV replication. However, the binding partners of Nsp9 have not been identified. In this study, seven host proteins were identified as Nsp9-binding proteins using yeast two-hybrid (Y2H). Among of them, we confirmed the interaction of Nsp9 with Annexin A2 (ANXA2) using Y2H, Co-immunoprecipitation (Co-IP), GST pulldown and immunofluorescence assay (IFA). We found that only full-length ANXA2 could bind with Nsp9 *in vitro* and Nsp9 interacted with endogenous ANXA2 in PRRSV-infected MARC-145 cells. In addition, we found that the Nsp9–ANXA2 interaction was partially reduced by RNase A treatment. Furthermore, PRRSV growth was significantly hindered in ANXA2-knockdown MARC-145 cells. Taken together, these results indicate that Nsp9 binding partner ANXA2 is beneficial for PRRSV replication.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), one of the most important porcine pathogens, causes large economic losses to the pig industry worldwide (Feng et al., 2008; Neumann et al., 2005; Pejsak et al., 1997). PRRSV belongs to the genus Arterivirus within the family *Arteriviridae*, which includes equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). The PRRSV genome is a single-strand positive RNA which is approximately 15 kb in length, encoding ten overlapping open reading frames (ORFs) and contains 5′ and 3′ untranslated regions (UTR) (Snijder et al., 2013). The non-structural proteins (Nsps), encoded in the 5′-proximal two-thirds of the genomic RNA, are synthesized as polyproteins from two ORFs, ORF1a and ORF1b. Once translated, the two polyproteins are processed to yield 14 different Nsps: including Nsp1α, Nsp1β, Nsp2-6, Nsp7α, Nsp7β, and Nsp8-12 (Fang and Snijder, 2010). It has been demonstrated that some of the Nsps are assembled to form the viral replicase complex with host cell components. Nsps are also involved in viral RNA replication, subgenomic mRNA transcription, and translation.

RNA-dependent RNA polymerase (RdRp) is required, in concert with other viral and host proteins, for viral replication and transcription in all positive-strand RNA viruses (O'Reilly and Kao, 1998). Nsp9 is the only RdRp of PRRSV which is essential for viral RNA replication (van Marle et al., 1999). The conserved GDD motif within the RdRp domain plays a major role in RNA amplification (Zhou et al., 2011). Previous studies have demonstrated that host factors can participate in positive RNA (+RNA) viral replication. For example, Hepatitis C virus (HCV) RdRp interacts with the cellular RNA helicase p68, which is required for HCV genomic negative-strand synthesis (Goh et al., 2004). Recently, it has been demonstrated that Japanese encephalitis virus (JEV) RdRp interacts with host protein Hdj2 and the interaction is necessary for promoting viral RNA synthesis and increasing viral titer (Wang et al., 2011). Interestingly, host Cyclophilins A (CyPA) and Cyclophilins B (CyPB) can enhance HCV RdRp transcription at the early stages and thereby promote viral replication (Weng et al., 2012). However, the host proteins interacting with PRRSV RdRp have not been reported.

Annexin A2 (ANXA2) belongs to the annexin family, which are structurally related calcium- and phospholipid-binding proteins.

* Corresponding author. Tel.: +86 189 4606 6289; fax: +86 451 5199 7170.
E-mail address: wengcj@hvri.ac.cn (C. Weng).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.virusres.2014.05.015
0168-1702/

Most Annexin proteins consist of two principle domains, the variable N-terminal domain and the conserved C-terminal domain which harbors the calcium- and membrane-binding sites (Gerke et al., 2005). ANXA2 is highly expressed in eukaryotic cells and localizes in the cytosol and on the membrane in non-stimulation status (Saxena et al., 2012). Previous reporters showed that ANXA2 implicated in the replication of certain viruses. For example, ANXA2 can facilitate the formation of the viral RNA replication complex and is required for efficient virus assembly (Backes et al., 2010; Saxena et al., 2012). ANXA2 can bind with capsid protein VP1 of Enterovirus 71 (EV71) and enhance viral infectivity (Yang et al., 2011). Interestingly, Zhang et al. found that ANXA2 can be incorporated into virions from PRRSV-infected cells (Zhang et al., 2010), however the function of ANXA2 in the PRRSV life cycle remains unknown. In this study, we identified cellular ANXA2 as a novel interacting partner of PRRSV Nsp9 and showed that ANXA2 is beneficial for PRRSV replication *in vitro*.

2. Materials and methods

2.1. Cells, viruses, and antibodies

MARC-145 cells and human embryonic kidney 293 cells (HEK293) were grown in GIBCO™ Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere containing 5% CO_2. The viral strain used in this study was HP-PRRSV HuN4 (Tong et al., 2007) with a titer of $10^{5.5}$ $TCID_{50}$/mL.

Mouse anti-Flag monoclonal antibody (mAb) and rabbit anti-Flag polyclonal antibody (pAb) were purchased from Sigma–Aldrich. Mouse anti-HA mAb and rabbit anti-HA pAb were purchased from Cell Signaling Technology. Rabbit anti-ANXA2 pAb was purchased from Proteintech Group Inc. Mouse anti-Nsp9 mAb was kindly provided by Prof. Hanchun Yang (College of Veterinary Medicine, China Agricultural University). Horseradish peroxidase (HRP)-conjugated goat anti-mouse pAb and HRP-conjugated goat anti-rabbit pAb were purchased from Sigma–Aldrich. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse mAb and tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-rabbit were purchased from Santa Cruz Biotechnology.

2.2. Plasmid construction

The Nsp9 gene was amplified by RT-PCR, using HP-PRRSV HuN4 RNA as the template, and then cloned into the pGBKT7 and pFlag-CMV-4 vectors; the resultant plasmids were named as pGBKT7-Nsp9 (BD-Nsp9) and pFlag-Nsp9, respectively. The porcine ANXA2 gene was amplified by RT-PCR, using total RNA of pulmonary alveolar macrophages (PAMs) as the template, and then cloned into vector pCAGGS-HA (pHA) vector, which was generated by introducing sequences encoding the HA-tag into vector pCAGGS. The resultant plasmid expressing N-terminal HA-tagged ANXA2 protein was named as pHA-ANXA2. A series of ANXA2 and Nsp9 truncated mutants were generated from pHA-ANXA2 and pFlag-Nsp9 by conventional PCR techniques. All primers are listed in Table 1. The ANXA2 cDNA was also cloned into vector pGADT7 vector or pGEX-6p-1 vector, named as pGADT7-ANXA2 (BD-ANXA2) or pGEX-6p1-ANXA2 (pGST-ANXA2), respectively. All plasmids were verified by sequencing.

2.3. Yeast two-hybrid screen

The Matchmaker™ Gold Yeast Two-Hybrid System (Clontech) was used to screen the host proteins that interact with Nsp9. A cDNA library was prepared in pGADT7-Rec (Clontech) using RNA derived from PAMs and then transformed into yeast strain Y187 (Clontech), named as pretransformed library. The bait plasmid pGBKT7-Nsp9 was transformed into yeast strain Y2H (Y2H/BD-Nsp9), and transformants were selected on SD plates lacking tryptophan. Y2H/BD-Nsp9 was subsequently mated with pretransformed library, and putative interacting clones were selected on quadruple-dropout plates lacking adenine, histidine, tryptophan and leucine (SD/-4) for 1 week. Selected clones were replated on quadruple-dropout plates containing 5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside (X-α-Gal) and Aureobasidin A (SD/-4/X-α-Gal). Colonies that turned blue within 24 h were chosen as positive colonies and cultured for plasmids extraction. Plasmids were amplified in *Escherichia coli* Top10 competent cells, and target insertions were verified by sequencing and NCBI BLAST analysis. To confirm the positive results, the respective bait and prey plasmids were co-transformed into the yeast strain Y2H Gold and retested on SD/-4/X-α-Gal plates again. Prey isolated from blue colonies in the absence of the Nsp9 bait, representing false positives, were eliminated. To identify the interacting region between Nsp9 and ANXA2, a series of constructs encoding successive truncations of ANXA2 was generated and then interactions between Nsp9 and truncated ANXA2 proteins were tested using Y2H and Co-IP.

2.4. Co-immunoprecipitation (Co-IP)

HEK293 cells were transfected with the indicated plasmids using X-tremeGENE HP DNA Transfection Reagent (Roche). Transfected cells were collected at 48 h after transfection. The cells were washed one time with 1X cold phosphate-buffered saline (1XPBS [pH 7.4]), and lysed for 30 min at 4 °C in cell lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM $MgCl_2$, and 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml protease inhibitor cocktail (Roche). Detergent-insoluble materials were removed by centrifugation at 15,000 × *g* for 20 min at 4 °C. The supernatants were transferred to new tubes and incubated with anti-Flag (M2) antibody-conjugated agarose beads (Sigma) for 4–8 h at 4 °C. For detection of the interaction between endogenous host protein and Nsp9 in PRRSV-infected cells, MARC-145 cells were grown on 10 cm diameter dishes and infected with PRRSV at a multiplicity of infection (MOI) of 0.1 for 24 h. Cells were lysed in 600 μL of lysis buffer, and the supernatants were pre-cleared with protein A/G-agarose beads. The supernatant was incubated with 2 μg of anti-Nsp9 mAb for 4–8 h, and then 20 μL of protein A/G-agarose beads was added, followed by an additional 1 h of incubation. Immunoprecipitation pellets were gently washed four times with 500 μL of lysis buffer, boiled in SDS sample buffer, subjected to 12% SDS-PAGE gel, and immunoblotted with antibodies as indicated.

2.5. Western blotting

Cell lysates were prepared and the protein concentration estimation was performed with the bicinchoninic acid protein assay reagent (Pierce) according to the manufacturer's instructions. 20–40 μg samples were loaded per lane and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked for 1 h in blocking buffer (5% skimmed milk, 0.1% Tween-20 in PBS) before incubation for an hour in primary antibody diluted in blocking buffer. Membranes were washed three times with washing buffer (0.1% Tween-20 in PBS), and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) for an hour. Samples were reacted with SuperSignal reagent (Pierce).

Please cite this article in press as: Li, J., et al., The interaction between host Annexin A2 and viral Nsp9 is beneficial for replication of porcine reproductive and respiratory syndrome virus. Virus Res. (2014), http://dx.doi.org/10.1016/j.virusres.2014.05.015