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A conjugate protein containing HIV TAT, ISG20, and a PRRSV polymerase binding inhibits PRRSV replication and may be a novel therapeutic platform

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS), which is caused by Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection, has caused substantial economic losses for the global swine industry. To date, there are limited commercially available measures to control the spread of PRRSV. The available vaccines are unstable and there is no anti-PRRSV therapeutic available. Therefore, this study designed a novel recombinant antiviral protein that included a novel polypeptide that binds to the PRRSV polymerase (p9), the transduction ability of the HIV TAT, and the nucleotide degradation activity of interferon stimulated gene 20 (ISG20). The recombinant proteins TAT-p9-ISG20 and p9-ISG20 were expressed in MARC-145 cells by transient transfection and then tested for antiviral activity and entry efficiency. The p9-ISG20 construct had greater antiviral activity than either p9 alone (1.37-fold) or ISG20 alone (1.9-fold). Addition of the HIV TAT protein increased the entry efficiency of p9-ISG20 by 1.57-fold, which was associated with increased anti-viral activity (1.52-fold) compared to P9-ISG20. In summary, TAT-p9-ISG20 achieved a synergistic effect by combining three different antiviral proteins and may be a novel PRRSV therapeutic platform.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV). PRRSV belongs to *Arterivirus* which display a complex replication and immune escape mechanisms. *Arterivirus* infect pigs (PRRSV), horses (Equine arteritis virus, EAV) and simian (Simian Hemorrhagic Fever Virus, SHFV) (Archambault et al., 2014; Snijder et al., 2013). These viruses, especially PRRSV, hazard animal health. PRRS has resulted in substantial economic losses for the swine industry in China (Li et al., 2007). PRRSV control is challenging due to the prevalence of subclinical infections and a high pathogen-carrier rate. The commercially available vaccines are unstable and there is not PRRSV specific therapy on the market (Jeong et al., 2016; Renukaradhya et al., 2015). Therefore, there is an urgent need to find effective methods to control PRRS (Corzo et al., 2010; Perez et al., 2015).

Previously, our study described 15 peptides that inhibit the function of the PRRSV polymerase and helicase proteins (Liu et al., 2012). These peptides were screened from phage display library with 9 random amino acid elements. One of these, p9 (HRILMRIRQMMT) showed strongest inhibitory effects on PRRSV replication *in vitro*. P9 peptide was selected from the phage plaques target to PRRSV polymerase protein. This peptide showed high antiviral activity against PRRSV *in vitro*. Based on physico-chemical property analysis, p9 may be degraded rapidly due to metabolic clearance, resulting in a short half-life *in vivo*. Moreover, p9 has a high specificity for the PRRSV polymerase and selectively binds to polymerase. Long-term treatment with p9 may lead to the emergence of resistant PRRSV strains through mutations. These impede the application of p9 antiviral peptide in clinical. Therefore, a complementary anti-PRRSV agent based on p9 may be a usefully and firmly select in PRRSV clinical medication (Sun et al., 2014; Wang and Zhang, 2014; Wang et al., 2013).

The IFN-stimulated gene 20 kDa protein (ISG20) is an interferon regulated RNase that degrades viral RNA. ISG20, along with ISG20L1 and ISG20L2, are members of the yeast RNA exonuclease 4 homolog (REX4) subfamily (Espert et al., 2003; Zhou et al., 2011). All of the

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REX4 family members are either RNases or DNases, and prefer single-stranded RNA over single-stranded DNA (Nguyen et al., 2001). In antiviral studies, ISG20 inhibited Encephalomyocarditis virus, vesicular stomatitis virus, influenza virus, and Human Immunodeficiency Virus by degrading viral RNA (Espert et al., 2003; Espert et al., 2005). ISG20 also restricted the replication of hepatitis B virus (HBV) RNA (Hao and Yang, 2008; Lu et al., 2013). ISG20 has also been reported to inhibit PRRSV, suggesting it might digest PRRSV RNA thereby inhibiting PRRSV replication. We hypothesized that the anti-PRRSV effects of ISG20 may be related to the PRRSV polymerase, which is critical for PRRSV replication and transcription. Alternatively, ISG20 may recognize PSSRV RNA bound to the polymerase. Thus, ISG20 is a suitable candidate agent complementary with p9.

To develop a practical antiviral agent against PRRSV, this study designed a novel recombinant protein that conjugated p9 to EGFP and ISG20 (p9-Linker-EGFP-Linker-ISG20) called P9-ISG20. We hypothesized that the p9-ISG20 conjugate would have greater anti-PRRSV activity than p9 alone due to the ability of p9 to target ISG20 to the PRRSV RNA. It is a new approach to develop synergism antiviral agent by targeting viral replication system.

2. Materials and methods

2.1. Cells and plasmid

MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco). MARC-145 is Simian kidney source cell line derived from Chinese academy of sciences with no mycoplasma and bacterial contaminate. All cells used in this study were 2 to 10 passages. The genes encoding p9, EGFP and monkey ISG20 were synthesized (Genscript Co. LTD). The genes for P9, monkey ISG20, and PRRSV polymerase (SY0608 strain) were cloned into the pEGFP N1, pmCherry N1, and pFlag 7.1 vectors and used for overexpression or co-localization analyses. The genes for HIV TAT, p9, and ISG20 were cloned into the pEU-01 vector and expressed in a cell free system (TIANGEN Biotech).

2.2. Real time RT-PCR and TCID50

To determine whether p9-ISG20 had antiviral activity, plasmids containing the p9-ISG20 genes were transfected into MARC-145 cells. Twenty-four (24) h after transfection, the cells were infected with PRRSV (0.1 multiplicity of infection [MOI]). The cells were collected for real time PCR analyses 24 h post infection. Real time RT-PCR was performed as previously described (Liu et al., 2012). Briefly, total RNA was reverse transcribed using the PrimeScriptTM Kit (Takara) according to the manufacturer's instructions. GAPDH was used as an internal control. The synthesized cDNA samples were used for real time PCR analysis, which was performed using a StepOne Real-Time PCR System (ABI) with SYBR Premix Ex TaqTM (Takara) according to the manufacturer's instructions. The relative fold changes in gene expression after treatment were calculated using the $2^{-\Delta\Delta CT}$ method. The specific primer pairs used in the PCR reactions are shown in Table 1. Results are expressed as relative changes in gene expression normalized to GAPDH CT values. The $TCID_{50}$ was determined by Reed-Muench method as previously described (Liu et al., 2012). Briefly, MARC-145 cells were treated with peptide and inoculated with PRRSV. 24 h after inoculation, the supernatant was collected and frozen at − 70 °C until use. Serial 10-fold dilutions were made from the supernatant stocks, and 100 μl samples of each dilution were added to duplicate wells of a 96-well plate containing a 60% confluent monolayer of MARC-145 cells. Five days were allowed for the appearance of cytopathic effect (CPE). The dilution causing cytopathology in half of the cultures (the median tissue culture infective dose, TCID50) was then calculated as described by the Reed and Muench method.

Table 1
Primers for real time PCR.

Real time PCR primers	Sequences (5′-3′)
PRRSV F	AGTGGGTCGGCACCAGTT
PRRSV R	GCAGACAAATCCAGAGGCTCAT
MARC-145 actin F	CAGCACGATGAAGATCAA
MARC-145 actin R	GGGTGTAACGCAACTAAG
PRRSV (+)RNA RT primer	ATCGTCGTCGTAGGCTGCTCCTCCCAGTCCTCACCATT
PRRSV (+)RNA F	CTCGCAGTCACCCCTTAT
PRRSV (+)RNA R	ATCGTCGTCGTAGGCTGCTC
PRRSV (−)RNA RT primer	GTATCGCTGGACACTGGACCGGAGGATTACAATGATGCGT
PRRSV (−)RNA F	GTATCGCTGGACACTGGACC
PRRSV (−)RNA R	AAAGTTGGTTCAATGACAGG

2.3. Co-localization and viral replication assessed by immunofluorescence

The level of PRRSV N protein infection was used to assess viral replication following treatment with p9-ISG20. MARC-145 cells were seeded in 6 T cell plate with glasses. When the cells growth to 50% confluent monolayer, cells were treated with p9-ISG20 or control, and then were fixed in 80% ice-cold acetone at − 20 °C for 10 min to fix and perforate cell. The cells were then stained with antibody directed against the PRRSV N protein (SDOW17 Monoclonal antibody) and 4,6-diamidino-2-phenylindole (DAPI). The cells were washed and fluorescence was visualized using a Nikon confocal microscope (C2).

2.4. Bimolecular Fluorescence Complementation Assay

A bimolecular fluorescence complementation (BiFC) assay was also performed as previously described (Liu et al., 2012). Sequences encoding the amino-(residues 1 to 173, VN) or carboxyl- (residues 174 to 239, VC) fragments of Venus were fused to the C-terminus of the full-length or truncated PRRSV N proteins *via* a 3 × (GGGGS) linker (Shimozono and Miyawaki, 2008; Shyu et al., 2006). The helicase gene was amplified from PRRSV (SY0608) and fused to the N-termini of VN and mutant p9-ISG20, respectively or to the VC for use as negative controls. MARC-145 cells were cultured in 6-well plates until they reached approximately 60% confluence and then co-transfected with 500 ng of each BiFC plasmid using Lipofectamine 2000 Transfection Reagent. At 14 h post-transfection, the cells were collected and imaged.

2.5. Fluorescence in situ hybridization

In brief, cells were co-transfected with p9-EGFP-ISG20 and polymerase-mCherry plasmids for 24 h. After transfection, the cells were infected with PRRSV (0.1 MOI). Twenty-four (24) h later, samples were collected and a DIG-labeled GP7 probe was used to detect intracellular PRRSV RNA.

2.6. Transfection efficiency measurements

Large proteins conjugated to the HIV TAT protein transduction domain have a higher transfection efficiency than the wild type proteins (Debaisieux et al., 2012). Therefore, the HIV TAT transfection domain is conjugated to p9-ISG20. Protein products from TAT-p9-ISG20 and p9-ISG20 production systems were purified, quantified, sterilized, and used to treat MARC-145 cells. After incubating the cells for 1 h at 37 °C, they were washed three times with PBS and the EGFP fluorescence intensity was measured using a multifunctional microplate reader.