Antiviral activity of phage display selected peptides against Porcine reproductive and respiratory syndrome virus in vitro

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ABSTRACT

Porcine reproductive and respiratory syndrome is an important infectious disease of pigs and has a significant harmful effect on the livestock industry, especially in China. PRRSV ORF1b gene encodes primary proteins which play a vital role during PRRSV replication. In this paper, various 12-amino-acid peptides were displayed. These peptides could bind to the polymerase and helicase of PRRSV ORF1b protein, respectively, in which p9 exerted the highest antiviral activity with an IC50 of 56 μM, and the minimum toxicity to cells. It was proved that p9 inhibited PRRSV replication in infected MARC-145 cells in a dose-dependent manner, and the amino acid sequence of HRILMRIR was important for antiviral activity of p9. Also, p9 could bind to the cell membrane and penetrated into cells. These results suggested that p9 might be a potential therapeutic drug for PRRSV infection.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV), and is a widespread infectious disease of pigs (Meulenberg et al., 1993). At the present time, PRRSV is known to damage the immune system of pigs, and the effect of commercial vaccines to prevent PRRSV infection is limited (Christopher-Hennings et al., 1995; Mateu and Diaz, 2008). Therefore, it is urgent to explore new antiviral drugs to control the PRRS.

PRRSV is divided into two major genotypes: the European (EU; type 1) genotype and the North American (NA; type 2) genotype (Meng et al., 1995), which is the main type isolated in China. PRRSV is a single-stranded positive-sense RNA virus of about 15 kbp in length. The genome has a cap structure at its 5'-end and a poly(A) tail at its 3'-end and encodes nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, ORF3–ORF7). ORF1 and ORF2 encode virus replication-related proteins, and account for 80% of the virus genome (Mounir et al., 1995; Nelson et al., 1995). ORF1a and ORF1b encode a polyprotein, which is proteolytically processed to 12 nonstructural proteins (NSPs). Among these, ORF1b encoded proteins are RNA-dependent RNA polymerase (NSP9), helicase (NSP10) and the conserved C-terminal domain (CTD) (NSP11) (Fang et al., 2004). These proteins are important factors in the process of virus infection (Castel et al., 2011; Sergeeva et al., 2006). Therefore, ORF1b can be used as a potential target for antiviral drug screening.

Phage technology can be used to carry out high-throughput screening of functional polypeptides, and the polypeptide gene cloning number in phage peptide libraries can reach more than a million. This technology has been applied increasingly in drug development trials, and it is an effective method for the development of new drugs (Bai et al., 2006; Hall et al. 2009). At present, many antiviral peptides have been obtained by phage screening technology, such as inhibition polypeptides of infectious bronchitis virus (IBV) (Peng et al., 2006), human immunodeficiency virus (HIV) (Welch et al., 2010), hepatitis C virus (HCV) (Hong et al., 2010), hepatitis B virus (HBV) (Ho et al., 2003), Newcastle disease virus (NDV) and other viruses (Chia et al., 2006). Some of these polypeptides have reached the experimental stage. This experiment uses a 12-peptide phage library to screen 12 peptides for activity against PRRSV ORF1b RNA polymerase and helicase protein. One peptide was found to interact with the PRRSV polymerase and suppress virus replication effectively.

Results

Identification of displayed peptides with PRRSV ORF1b protein

Proteins encoded by PRRSV ORF1b play vital roles during PRRSV replication. To design inhibitors based on these proteins...
were predicted with the ProtParam algorithm. The molecular weight (MW), pI, and grand average of hydropathicity (GRAVY) were calculated with the ProtParam algorithm.

Inhibitions of peptides on PRRSV replication in vitro

The inhibition ability of synthetic peptides following phage display was determined by real-time PCR. MARC-145 cells were inoculated with viruses at 0.01 MOI for 1.5 h at 37 °C, and treated with or without peptides at 500 μM for 24 h, and cells treated with DMSO were used as the negative control. The results showed that p9 peptide exerted the highest antiviral activity among that of peptides with PRRSV polymerase in vitro (Fig. 1).

The results of Real-time PCR and TCID50 showed that p9 was the greatest antiviral peptide among all these selected peptides. Other peptides such as p2, p3 and p13 also showed high antiviral effects. Furthermore, 500 μM p9 exerted higher antiviral activity than that of 10 μM ribavirin (Ibarra and Pfeiffer, 2009).

Antiviral activity of p9

To further investigate the antiviral activity of p9, the toxicities of p9 to MARC-145 cells were detected using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The results showed that CC50 and IC50 of p9 (24 h) to MARC-145 cell was 473.00 μM and 56.47 μM, respectively, and CI (CC50/IC50) of p9 was 8.38 (Fig. 2a). When the concentration of p9 was 250 μM, the ratio of virus copy number/cell reference copy number was 0.8625, and cell survival rate was 74.54%. The results of TCID50 are the same as real-time results (Fig. 2b).

To study the time- and dose-dependent effect of p9 on inhibition of PRRSV replication, MARC-145 cells were incubated with p9 from 31.25 μM to 1000 μM for the indicated periods at 37 °C. It has been reported that a complete cycle of PRRSV replication is 24 h (Cafruny et al., 2006). As shown in Fig. 2c, during PRRSV replication (12 h), 1000 μM, 500 μM and 250 μM p9 showed significant inhibition on PRRSV replication. After 24 h, p9 exerted inhibition on intracellular PRRSV numbers of MARC-145 cells in dose-dependent manner. Furthermore, during the second replication cycle (36 h and 48 h), with increasing doses of p9, the intracellular PRRSV numbers of MARC-145 cells treated with p9 were gradually decreased. Furthermore, cytotoxicity in these two points was very low (data not shown). These results suggested that p9 inhibits PRRSV replication in a dose-dependent manner.

To detect the intracellular retention time of p9 which is a potential important antiviral factor, MARC-145 cells were treated with rhodamine-labeled p9. Results of light intensity/scanning area of fluorescence gray scanning showed that the retention time of p9 in cells was at least up to 144 h, and at 24 h, the fluorescence intensity of rhodamine-p9 is highest among all indicated times. However, at 48 h and 72 h, the intensities of rhodamine-p9 were decreased, compared to that of 24 h. Subsequently, fluorescence levels were dramatically reduced, and were almost not observed after 96 h (Fig. 2d).

To further study the inhibition effects of p9, p9 was added for 4 h before and after virus infection and at 0 h after virus infection, respectively (Fig. 2e). The results of Real-time PCR showed that at 24 h, the intracellular PRRSV numbers in PRRSV-infected MARC-145 cells pre-incubated with p9 for 4 h or 0 h were dramatically decreased, compared to that of DMSO control. Although PRRSV RNA of MARC-145 cells treated with p9 after PRRSV infection were lower than that of DMSO control, it was significantly higher than that of pre-incubated with p9 for 4 h or 0 h. Also, results at 48 h were similar that of 24 h. These results suggested that p9 stimulation before or together with PRRSV infection could strongly inhibit PRRSV replication in MARC-145 cells.

P9 penetrates cells

As an antiviral peptide, we detected penetration of p9 in MARC145 cells. Results showed p9 could adsorb to cell membranes in a short time (2 h), and could penetrate into the cytoplasm within 24 h. These results were observed in other cells too (data not shown) (Fig. 3).

### Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Target</th>
<th>Amino acid sequence</th>
<th>MW</th>
<th>pI</th>
<th>GRAVY</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
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</tr>
<tr>
<td>P3</td>
<td>Helicase</td>
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<tr>
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<td>Helicase</td>
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<tr>
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<td>Helicase</td>
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<td>1462.8</td>
<td>12.02</td>
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<td>HRLMRIRQMKT</td>
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<td>-1.808</td>
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<tr>
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<td>Polymerase</td>
<td>RMRKSKRIRFTR</td>
<td>1588.9</td>
<td>12.60</td>
<td>-2.175</td>
</tr>
</tbody>
</table>

| C1 | Control | SPRQJMHHMPKP | |
| C2 | Control | QRIRRTQFQPRMR | |
| C3 | Control | QMMRMIMMMRMRT | |
| C4 | Control | PRPRKMMMQGMK | |
| C5 | Control | HIIHRQIGTRP | |

The molecular weight (MW), pI, and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm.

To further investigate the antiviral activity of p9, the toxicities of p9 to MARC-145 cells were detected using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The results showed that CC50 and IC50 of p9 (24 h) to MARC-145 cell was 473.00 μM and 56.47 μM, respectively, and CI (CC50/IC50) of p9 was 8.38 (Fig. 2a). When the concentration of p9 was 250 μM, the ratio of virus copy number/cell reference copy number was 0.8625, and cell survival rate was 74.54%. The results of TCID50 are the same as real-time results (Fig. 2b).

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Fig. 2. P9 inhibition of PRRSV replication: (A) real-time PCR and MTT method were use to detect P9. IC50= 56.47, CC50 = 473. (B) Antiviral activity of p9 determined by TCID50, 500 μM p9 was added and CPE was recorded at 48 h. (C) Determination of the time–effect and dose–effect relationships of p9 on inhibition of virus replication, the experiment measured the inhibition efficiency within 12–48 h. (D) Fluorescence observation of duration of rhodamine-labeled p9 in cells, experiments were determined within 24–144 h. (E) Determination of the inhibition efficiency of p9 added at 4 h before and after virus infection, and carried out 24 h and 48 h (p < 0.05, indicated by *; p < 0.01, indicated by **).
Peptide inhibition drugs play an antiviral role based on their amino acid structure (Castel et al., 2011). To study the structure–activity relationship of p9, the amino acid in p9 was individually replaced by alanine (A) (Cunningham and Wells, 1989). As shown in Table 2, analysis results showed that although the molecular weights and isoelectric points of these mutated peptides were almost not changed, the solubilities were affected with the substitution of different positions of amino acids. For example, the solubilities of these mutated peptides with substitution of the 1st, 2nd, 6th, 8th or 12th amino acid were decreased, while of 3rd, 4th, or 7th amino acid were increased, respectively, and other replacements were little changed. Compared with the inhibition activity of these mutant peptides, the results showed that the antiviral activities of only the mutated peptides with the replacement of 3rd or 9th amino acid were increased, and other mutations had little effect on the activity and even reduced the activity. These results suggested that the solubility of peptide was not directly related to its antiviral activity.

Additionally, the CC50 and IC50 values of these mutants were measured, which represent the concentration of drug that is required to kill 50% of the cells in an uninfected culture, and represents the concentration of a drug that is required for 50% inhibition of viral replication in vitro, respectively. The results of CC50 values showed that only the mutated p9 with 12th amino acid was cytotoxic. Also, the IC50 values of 3-p9 and 9-p9 were lowest among all these mutated p9, which was 34.18 and 34.14, respectively, indicating that both amino acid positions were directly involved in the antiviral activity of p9 (Table 2).

To further study the relationship of the 3rd and 9th amino acid with p9 antiviral activity, p9 was truncated into three fragments, including a-p9, b-p9 and c-p9. The results showed that the IC50 value of b-p9 was lowest among these three truncated p9, suggesting the strongest the antiviral activity. Also, the solubility of b-p9 was hydrophilicity, whereas a-p9 and c-p9 were hydrophobic. These results suggested that b-p9 with sequence HRILMIRIR might be important for antiviral activity.

It has been reported that the enzymolysis and biological activity of dextral and levorotatory proteins are different. To study the discrimination of p9 chirality on antiviral activity, MARC-145 cells were treated with or without the dextral p9 (D-p9) and levorotatory p9 (L-p9) (Fig. 4). The results showed that the IC50 value of D-p9 was 16.12, which was lower than that of L-p9 (56.47), suggesting that the antiviral activity of D-p9 might be higher than that of L-p9 (Fig. 4a).

To compare antiviral activities of D-p9 with L-p9, the concentrations of 250 μM and 125 μM were used to detect the intracellular PRRSV numbers of MARC-145 cells. The results showed that the intracellular PRRSV numbers of MARC-145 cells with D-p9 treatment were significant lower than that of L-p9 treatment at 24 h, suggesting that the antiviral activity of D-p9 might be higher than that of L-p9 (Fig. 4b).

Circular dichroism spectroscopy was used to scan p9 at 210–500 nm wavelengths. A negative peak (−97.1972) at 245 nm and some miscellaneous peaks at 240–210 nm were obtained (Kumar et al., 2008). In circular dichroism spectroscopy assay, a negative peak at 245 nm represents a four-chain structure material in the assay sample. Circular dichroism spectroscopy assay of p9 showed the peptide has a positive peak at 265 nm and a negative peak at 245 nm. P9 did not fully comply with the profile of a standard four-chain structure that, and its structure was complex (Fig. 4c).

**P9 interacts with the PRRSV polymerase in cells directly**

To determine whether P9 interacts with the PRRSV polymerase intracellularly, we employed a newly developed technique, known as bimolecular fluorescence complementation (BiFC). BiFC is based on the principle that two interacting proteins individually fused to the N- and C-terminal fragments of a fluorescent protein can bring the two halves of the fluorescent protein into close, thereby facilitating reconstitution of the fluorophore. BiFC assays allow for the visualization and localization of specific protein–protein interactions within living cells.

**Table 2**
Modified of p9 sequence (IC50 and CC50 were shown in table).

<table>
<thead>
<tr>
<th>Modified peptide</th>
<th>Amino acid sequence</th>
<th>MW</th>
<th>pI</th>
<th>GRAVY</th>
<th>IC50 (μM)</th>
<th>CC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-P9</td>
<td>ARILMRIRQMMT</td>
<td>1519.9</td>
<td>12.30</td>
<td>0.217</td>
<td>716.4193</td>
<td>156.12263</td>
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<tr>
<td>2-P9</td>
<td>HAILMRIRQMMT</td>
<td>1500.9</td>
<td>12.00</td>
<td>0.325</td>
<td>Undetected</td>
<td>Undetected</td>
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<tr>
<td>3-P9</td>
<td>HRILMRIRQMT</td>
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<td>12.30</td>
<td>−0.425</td>
<td>34.18095</td>
<td>&gt;500</td>
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<tr>
<td>4-P9</td>
<td>HRILMRIRQMT</td>
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<td>12.30</td>
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<td>54.5872</td>
<td>&gt;500</td>
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<td>5-P9</td>
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The molecular weight (MW), pI, and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm.
We used Venus, a variant of enhanced yellow fluorescent protein, for the BiFC assays in our study. We first split the fluorescent protein between amino acid residues 173 and 174 to generate two fragments, VN and VC. As expected, neither VN nor VC alone produced a fluorescent signal when expressed in MARC-145 cells (data not shown). Likewise, co-expression of VN and VC also produced no fluorescence (Fig. 5b). We then generated full-length polymerase and helicase genes to fuse into the N-terminus of VN with a flexible linker and designated them VN-polymerase and VN-helicase, respectively. We generated p9 and mutant p9 gene to fuse into N-terminus of VC with a flexible linker and designated them VC-p9 and VC-mp9, respectively. In order to determine whether BiFC can efficiently detect the p9-polymerase interactions in living cells, cells were co-transfected with different combinations and examined for fluorescence. At 14 h post-transfection, we found that fluorescence, representing the p9-polymerase interaction, was observed in cytoplasm (Fig. 5b). The VN-helicase/VC-p9 and VN-polymerase/VC-mp9 pairs were employed as negative controls, when transfected into living cells neither produced positive BiFC signals (Fig. 5b).

Discussion

In recent years, PRRSV infection has seriously harmed the pig industry. PRRSV infection has caused great economic loss, especially in the years 2005–2008. To date, there is no effective drug that can control PRRSV infection, and PRRSV vaccination is ineffective. In this study, we expressed the key proteins involved in PRRSV replication and used phages display to screen for high-affinity peptides. A number of peptides, in particular p9, showed very high antiviral activity.

PRRSV polymerase is an RNA-dependent RNA polymerase and is an enzyme specific to viruses. Therefore, peptides screened by phages could play a role in inhibiting this specific virus enzyme in cells, and without affecting cell enzyme activity. RNA-dependent RNA polymerase is one of the most important proteins for intracellular replication of viruses, and its concentration can be determined directly by the copy number of the virus gene (Cheng et al., 2008). Therefore, this approach was used initially to evaluate the inhibition ability of peptides directly by detection of the intracellular virus copy number. Nearly all our selected peptides showed antiviral ability. Among these peptides, the inhibition ability of p9 was the strongest of the peptides tested. Therefore, p9 was selected as the peptide molecule for study. This study used real-time PCR to assess the inhibition ability of p9, namely to assess the inhibition ability of p9 on polymerase.

Peptide inhibitors can be degraded and metabolized by intracellular proteases after they enter the cell (Münch et al., 2007). Stability in cells was an important index to evaluate antiviral peptide. After viruses have invaded cells, they persist and form new virus particles, which results in transmission of infection from one cell to another. This process requires that virus inhibitors exist stably in cells for a prolonged time (Hinson and Cresswell, 2009; Wolf et al., 2010). Once virus replication has occurred, p9 inhibitory effect on viruses can act. Our study detected and observed the time of p9 peptide inhibition of viruses and the duration of p9 peptide in cells. Our results indicated that p9 at high concentrations could play an inhibitory role in cells for a prolonged period. Virus concentration in cells increased with time, but when compared with the PBS control group, the quantity of virus and speed of virus increase were both lower in the presence of p9. In an antiviral time–effect trial, detection of intracellular virus copy number at 72 h showed that inhibition effect of p9 could last up to 72 h (data not shown). Because of our chosen calculation method, we saw variation in virus copy number only in the first 48 h. The intracellular degradation and metabolic rate of p9 showed discontinuous variation. At 48 h, intracellular p9 concentration was high, and the corresponding virus replication was also inhibited strongly. After 48 h, fluorescence observation showed that p9 concentration was still high. At 96 h, p9 concentration was low as seen by fluorescence observation. We speculated the p9 was degraded or metabolized quickly (fluorescence gray scanning results) in the 48–96 h period. For the virus infected group in the corresponding 48–96 h, many cells died. Fluorescence observation of intracellular duration of p9

Fig. 4. Comparison of time–effect and dose–effect between levorotatory p9 (L-p9) and dextral p9 (D-p9): (A) L-p9 and D-p9 IC50 comparison, D-Pp IC50 = 16.12, L-p9 IC50 = 56.47. (B) Two concentrations of 250 and 125 M and two time points of 24 h and 48 h were selected. (C) CD spectrogram of p9 dissolved in DMSO; a stronger negative peak was visible at 245 nm (p < 0.05, indicated by *).
showed that p9 content at 96–144 h was at a lower level and decreased slowly.

We further tested the inhibitory effect of p9 added at 4 h before and after virus inoculation, which represented the virus prevention and the treatment effects of p9. The results showed that p9 added before cell infection had higher antiviral ability than p9 added at 0 h or 4 h after cell infection. In the +4 h group, p9 was added at 4 h after viruses had infected cells, and we calculated that p9 entered the cells probably at 5–6 h. In this 5–6 h period, viruses had enough time to complete intracellular capsid removal and polymerase expression (You et al., 2008; Cheng et al., 2008; Konduru and Kaplan, 2010). Based on these results, we suggest that p9 plays an inhibitory role, and that its inhibition efficiency was comparatively much lower than that of the /C0 4 h group and of that of the 0 h group. However, the inhibitory effect of p9 could still be detected.

The amino acid composition of p9 makes it weakly hydrophilic (GRAVY = –0.200). Compared with otherscreened peptides, it was in the mid range, i.e. the remaining peptides were more hydrophobic (0.833) or hydrophilic (–2.717), but their antiviral activity was less than that of p9. Adjacent amino acid mutations in p9 showed that the GRAVY properties of the mutant peptides had no correlation with antiviral activity. Both mutation to hydrophobic or hydrophilic residues resulted in either high or low activity. It was found that mutations at the third and ninth amino acid sites increased the antiviral activity of the peptide. We speculated that the two mutations to alanine sites could expose the active area of the peptide, which aided peptide and polymerase combination. To further study the active center positions of p9, we truncated p9 by removal of either the N-terminus end, C-terminus end or of both ends. The results showed that removal of four amino acids at the C-terminus end increased the activity of the peptide, while the activities of the other truncated peptides decreased in comparison. This finding indicated that the active area of p9 was mainly in the eight amino acids at the N-terminus end. Therefore, we speculated that the active center of p9 and the key amino acids that determined the activity were discontinuous (Bajorath et al., 2009; Wawer and Bajorath, 2009). This situation is similar to that found for B-cell epitopes, which are composed of discontinuous amino acids. Circular dichroism spectroscopy results showed that p9 had an obvious negative peak at 245 nm, but had no peak 265 nm, a finding which is possibly typical of peptides with more complex structures (Olga et al., 2009). However, there were many hybridized peaks at 240–220 nm, which were possibly caused by interference by the p9 solvent DMSO. We speculated that p9 does not form a fixed structure in DMSO, and that its status varied with environmental changes (Shen et al., 2000).

We compared dextral p9 (D-p9) and levorotatory p9 (L-p9) and found that the inhibition activity of dextral p9 was much higher than that of levorotatory p9, a finding that is in line with other research results (Zeng and Sabra, 2011). Dextral p9 proteins are degraded more slowly in vivo, which allows D-p9 to have a more effective inhibitory role.

Our BiFC experiments showed interaction between p9 and the PRRSV polymerase. These results demonstrate that p9 interacts with the polymerase itself rather than with PRRSV nucleic acid associated with the polymerase.

Our studies show that the PRRSV Polymerase is an attractive drug target. Furthermore, we found that p9 and p9 derivatives are potent antiviral peptide with low cytotoxic even at exceedingly high concentrations. Importantly, a few amino acid changes result in increasing the antiviral potency of p9. Thus, further development of p9 as an antiviral agent may lead to a new polymerase inhibitors expected to inhibit PRRSV.

**Fig. 5.** BiFC assay of p9 and polymerase interactions intracellular: (A) the design of fluorescence plasmids and (B) the fluorescence visualized in living cells. M-p9 was designed with p9 amino acid elements and used as a negative control.
Materials and methods

Cells and plasmid

MARC-145 cells, Vero cells, BHK-21 cells, and RK-13 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco). The two protein genes of polymerase and helicase in ORF1b of PRRSV (SY0608) were cloned into the pCOLD plasmid or the pET-32a(+) plasmid (Hayashi and Kojima, 2008), respectively.

Expression and purification

The plasmids containing three protein genes (polymerase or helicase) were transfected into Rosetta or R2 host bacteria, respectively, and these proteins were expressed after induction, and were purified with Ni affinity according to the manufacturer’s instructions.

Phage display library screening

Selection procedure was slightly modified following with the manufacturer’s protocol. Briefly, the Ph.D.-12TM Phage Display Peptide Library (New England Biolabs) was first exposed to plates coated with three purified recombinant PRRSV proteins, respectively. Unbound phages were washed away, and the bound phages were eluted and amplified for the second screening round. These phages that showed high binding activity to recombinant proteins by an indirect enzyme-linked immunosorbent assay were selected and sequenced from the fourth round selection, and then analyzed to determine the homology proteins using BLAST software (http://ncbi.nlm.nih.gov/BLAST/). All peptides were chemically synthesized by TASH Biotechnology Co. Ltd with purity of more than 95%, and were dissolved in DMSO.

Real-time polymerase chain reaction (PCR) and tissue culture infective dose (TCID50) assay

The effects on PRRSV of peptides treatment were determined with both real-time PCR and TCID50 methods. Simply, total RNA was prepared using TRizol reagent (Invitrogen) from cells treated with or without peptides or drugs. Reverse Transcription was performed following manufacturer protocols (Invitrogen). Briefly, MARC-145 cells were incubated in 96-well plates in 200 μl of DMEM. Incubation was terminated by aspirating the media and DTT solution (5 mg/ml in PBS) were added to each well. Formazane formation was terminated after 4 h by removing the DTT solution. Subsequently DMSO was added to each well to solubilize formazane, and the formazane-containing samples were measured at 590 nm in a microplate reader.

Fluorescence microscopy

MARC-145 cells were treated with or without peptide at various time points. After fixation in 80% ice-cold acetone at –20 °C for 10 min, cells were stained with 3,3-dioctadecylxylcarbocyanine perchlorate (DiO) for 3 min and stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. After washing, cells were detected and photographed with fluorescence microscopy.

Bimolecular fluorescence complementation (BiFC) assay

A variant of yellow fluorescent protein, Venus, was obtained by site-directed mutagenesis of pEYFP-N1 (BD-Clontech). As shown in Fig. 5a, sequences encoding the amino- (residues 1 to 173, VN) or carboxyl- (residues 174 to 239, VC) fragments of Venus were fused to the N-terminus of VN and mutant p9 (mp9) (LQMIRHRMTIMR) to the VC for use as negative controls. The primers used for BiFC are shown in Table 3. MARC-145 cells cultured in 6-well plates to about 60% confluency were co-transfected with 500 ng of each BiFC plasmid using the Lip 2000 Transfection Reagent. At 14 h post-transfection, live cells were visualized using an Olympus inverted fluorescence microscope.

Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
<th>Probe or sequences</th>
</tr>
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<tbody>
<tr>
<td>PRRSV</td>
<td>F: 5′–AGTGGGTCGCGCACCAGTT-3′</td>
<td>FAM–GTGACACACGACACAC-5′</td>
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<tr>
<td></td>
<td>R: 5′–GCTAGACAAATCCAGAGGCTCAT-3′</td>
<td>BHQ1</td>
</tr>
<tr>
<td>MARC-145 beta actin</td>
<td>F: 5′–CAGCACGATGAGAAGTCA-3′</td>
<td>FAM–CTGAAAACGACACAC-5′</td>
</tr>
<tr>
<td>VN-polymerase</td>
<td>F: 5′–TATAGATCTGAGGCGCGGATTGAGCCG-3′</td>
<td>BHQ1 Polymerase</td>
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<tr>
<td></td>
<td>R: 5′–GCGCTGACCAGCCCTCCCTGACAGAGTCAGTTTTCC-3′</td>
<td></td>
</tr>
<tr>
<td>VN-helicase</td>
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<td>Helicase</td>
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<tr>
<td></td>
<td>R: 5′–AGATCTCAGACATGACATGACACACACAGGCTCAGTGAC-3′</td>
<td>HRILMRQRQMMT</td>
</tr>
<tr>
<td>VC-p9</td>
<td>F: 5′–TTTTGGTTAGAGGAGGATGACGAC-3′</td>
<td>LQMIRHRMTIMR</td>
</tr>
<tr>
<td>VC-Mp9</td>
<td>F: 5′–ATAGACTGACTGACTGACGTATCTGACATGACGAC-3′</td>
<td>LQMIRHRMTIMR</td>
</tr>
</tbody>
</table>
Statistical analyses

All assays described here were repeated at least twice, and all the measurements were made in triplicate. Mean values ± standard deviation (SD) were calculated using Microsoft Excel. Statistical analysis was done by one-way analysis of variance and values were considered significant or when p < 0.05. Figures were performed using the GraphPad Prism 5.0 software.

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References


