Antiviral Research 109 (2014) 68–71

Contents lists available at ScienceDirect

Antiviral Research

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

Short Communication

Specific ligands for classical swine fever virus screened from landscape phage display library

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ARTICLE INFO

Article history:
Received 4 December 2013
Revised 12 May 2014
Accepted 18 June 2014
Available online 28 June 2014

Keywords:
Classical swine fever virus
E2 protein
Phage display
Specific ligand
Landscape phage display library
Antiviral activity

ABSTRACT

Classical swine fever (CSF) is a devastating infectious disease caused by classical swine fever virus (CSFV). The screening of CSFV-specific ligands is of great significance for diagnosis and treatment of CSF. Affinity selection from random peptide libraries is an efficient approach to discover ligands with high stability and specificity. Here, we screened phage ligands for the CSFV E2 protein from f8/8 landscape phage display library by biopanning and obtained four phage clones specific for the E2 protein of CSFV. Viral blocking assays indicated that the phage clone displaying the octapeptide sequence DRATSSNA remarkably inhibited the CSFV replication in PK-15 cells at a titer of 10^{10} transduction units, as evidenced by significantly decreased viral RNA copies and viral titers. The phage-displayed E2-binding peptides have the potential to be developed as antivirals for CSF.

Classical swine fever (CSF), a highly contagious and often fatal disease of swine caused by classical swine fever virus (CSFV), can lead to huge economic losses in the pig industry worldwide (Edwards et al., 2000). CSFV is a small enveloped virus with a single-stranded, positive-sense RNA genome of approximately 12.3 kb in length. The CSFV genome contains a single open reading frame encoding a polyprotein of 3898 amino acids that undergoes co- and post-translational processing by cellular and viral proteases, giving rise to four structural proteins C, E^{rns}, E1 and E2, and seven non-structural proteins N^{pro}, p7, NS2–3, NS4A, NS4B, NS5A and NS5B (Rümenapf et al., 1993; Meyers and Thiel, 1996; Tautz et al., 1997).

Despite that vaccines for CSF are commercially available, CSF remains a serious threat to the pig industry worldwide (Edwards et al., 2000; Leifer et al., 2013), due to the problem with the current CSF vaccines, the unavailability of robust and sensitive serological DIVA (differentiating infected from vaccinated animals) tools and the immunity gap. Therefore, it is necessary to develop novel antivirals to control CSF. The E2 envelope glycoprotein, which resides on the outer surface of the virion (Weiland et al., 1999), has been reported to be involved in the attachment and entry of CSFV (Hulst and Moormann, 1997; Wang et al., 2004). In addition, the E2 protein has also been proposed as a virulence determinant (van Gennip et al., 2004; Risatti et al., 2005). Therefore, the E2 protein is an interesting target for antivirals screening.

Phage display, a well-established powerful technology, has been widely used for ligand screening, disease diagnosis, drug development and biosensing (Samoylova et al., 2003; Dias-Neto et al., 2009; Mao et al., 2009; Wang et al., 2010; Qi et al., 2012; Lang et al., 2014). The f8/8 landscape phage library is a multibillion population of filamentous phage particles displaying random octapeptides on all the 4000 surface domain of the major coat protein pVIII (Petrenko et al., 1996). The high capacity and multivalence of this phage library makes it suitable for high-throughput screening of ligands that specifically bind with the given targets. In the past years, several peptides were identified as potential antivirals against avian influenza virus, Newcastle disease virus, human immunodeficiency virus, and hepatitis B virus using phage display technology (Ramanujam et al., 2002; Ho et al., 2003; Rajik et al., 2009; Welch et al., 2010).

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http://dx.doi.org/10.1016/j.antiviral.2014.06.012
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In this study, we attempted to use the phage display technology to screen specific peptides capable of binding to the E2 protein of CSFV and inhibiting the viral infection *in vitro*, demonstrating the potential of ligands as antivirals against CSFV.

Phage clones with the ability to bind the CSFV E2 protein that was expressed in *Pichia pastoris* (Fig. S1) were selected from the f8/8 landscape library (Petrenko et al., 1996) (Fig. S2) using a biopanning procedure. The amounts of input phages and eluted phages were determined by titration, and the phage recovery (eluted phages/input phages) in each round was calculated (Fig. S3). After three rounds of biopanning, eight phage clones were randomly selected, and the displayed nucleotide sequences were amplified by PCR (Petrenko et al., 1996) and sequenced. Four phage clones were obtained, each of which displayed a unique octapeptide (Table 1).

To verify the binding specificity of these phages with the E2 protein, two other structural proteins of CSFV, C and E^{rns}, which were expressed in *Escherichia coli* (Figs. S4–7), and a wild-type phage were included for both phage-ELISA and phage-capture assays. In the phage-ELISA, the four phage clones screened or the wild-type phage were coated on the wells of Immuno 96 Microwell plates (Nunc, Denmark). Then the biotinylated E2 protein was incubated with the immobilized phages, and the biotinylated E^{rns} and C proteins were used as controls. Thereafter, the wells were incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Thermo, USA), and finally *o*-phenylenediamine substrate (Aladdin, China) was added for color development. As expected, the four screened phages exerted significantly higher binding affinity with the E2 protein, compared with the C and E^{rns} proteins, while the control phage only gave low background signals with all the three proteins (Fig. 1). In phage-capture assays, wells of Immuno 96 Microwell plates were coated with the E2, E^{rns} or C proteins. Candidate phages and the wide-type phage (as control) were incubated with the coated proteins. Then the bound phages were eluted and titrated after washing away the unbound phages, and the phage recovery rate was calculated to compare the captured phages by different proteins. The selected phages bound to E2 at a significantly higher affinity than E^{rns} and C (Fig. 2A), and almost no wild-type phages were captured by the CSFV proteins. The results indicated that the four octapeptide-displayed phages did have specific binding to the E2 protein of CSFV. Different from the E^{rns} and C proteins that were expressed in a bacterial system, the E2 protein has a eukaryotic background, which was expressed in yeast. Thus, it would be interesting to compare the binding affinity of the phages with the E2 protein of different sources.

To investigate the binding performance of the E2-binding peptides displayed on the phages with CSFV, virus-capture assays were carried out. As shown in Fig. 2B, the clone E2P3 displaying the octapeptide GGSVPTET showed significantly higher binding affinity to CSFV, compared with the irrelevant octapeptide-displayed phage clones from the library.

To further determine whether the phages that displayed E2-binding peptides have the potential to inhibit the CSFV infection, we performed virus-blocking assays in PK-15 cells. Briefly, 200 $TCID_{50}$ CSFV was incubated with 10^8–10^{10} TU phages that displayed E2-binding peptides or irrelevant phage, and then

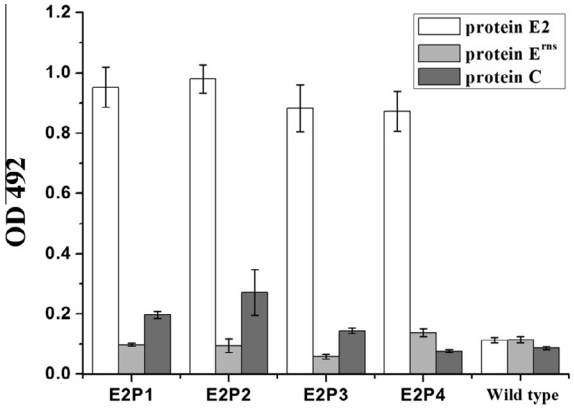

Fig. 1. Phage-ELISA for phage specificity. Four selected phage clones and the wild-type phage (served as a non-specific control) were respectively coated on the wells of Immuno 96 Microwell plates. Then the biotinylated E2 protein was incubated with the immobilized phages, and the E^{rns} and C proteins were used as controls. HRP-conjugated streptavidin was loaded to bind to the biotinylated protein, and finally *o*-phenylenediamine substrate was added for color development.

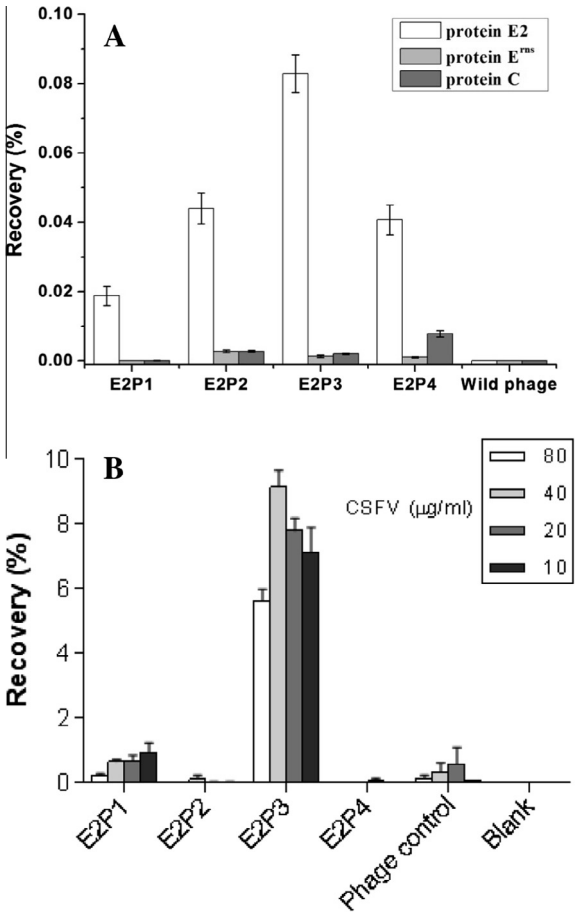

Fig. 2. (A) Phage-capture assay of E2-binding phages. Wells of Immuno 96 Microwell plates were coated with the proteins E2, E^{rns} and C. Candidate phages and the control phage were incubated with the coated proteins. Then bound phages were eluted and titrated after washing the unbound ones, and the phage recovery rate was calculated to compare the captured phages by different proteins. (B) Virus-capture assay of phages that display E2-binding peptides. 10^8 TU of E2-binding phages were used to capture 10–80 μg/ml of the purified CSFV, and an irrelevant octapeptide-displayed phage clone from the library was included as a negative control. Error bars represent standard deviation of three independent experiments.

Table 1
Amino acid sequences of the selected phages.

Phage clones	Peptide sequences
E2P1	DSRLEPNT
E2P2	ASRAPSST
E2P3	GGSVPTET
E2P4	DRATSSNA

inoculated into PK-15 cells, and the non-phage treated CSFV was included as control. After 72-h incubation, real-time RT-PCR was performed to determine the RNA copies of CSFV in different phage-treated cells. Both the cells infected with phage-treated viruses and mock-treated grew well without morphological difference, indicating that phages had no toxic effects on the cells. Virus-blocking assays showed that 10^{10} transduction unit (TU) of the clone E2P4 with the octapeptide DRATSSNA induced a marked reduction in the CSFV replication compared with the control phage that displayed an irrelevant octapeptide or non-phage treatment control (CSFV only) ($p < 0.05$), as evidenced by notably decreased viral RNA copies (Fig. 3A) using real-time RT-PCR (Zhao et al., 2008). At the same time, the titer profile of the infectious viruses in the cell cultures determined by viral titration was consistent with the genomic copies measured by real-time RT-PCR (Fig. 3B).

The E2 glycoprotein of CSFV has been proved to be involved in virus attachment and entry into target cells (Hulst and Moormann, 1997; Wang et al., 2004) and induction of neutralizing antibodies (Konig et al., 1995). Several antigenic regions and epitopes have been identified on the E2 protein of CSFV (van Rijn et al., 1994; Yu et al., 1996; Peng et al., 2008; Chang et al., 2010, 2012). Interestingly, the phage clone E2P3 exhibited binding affinity to CSFV with little antiviral activity against CSFV, whereas the phage E2P4 with low affinity to the virus effectively inhibited the replication of CSFV. This implies that binding affinity of phages does not necessarily associate with antiviral activity. A possible explanation is that the phage ligand mediates an inhibitory effect independent of virus binding, such as receptor blocking (Norkin, 1995). E2P4 is presumably to block viral infection by competitive binding to the receptor-binding site or neutralizing epitope, thereby inhibiting the virus attachment/entry. Conversely, E2P3 might bind a site on the E2 protein that is not linked to virus attachment/entry or the phage cannot exert enough spatial hindrance of virus attachment/entry, thus incapable of blocking viral replication. Another possible explanation is that the E2 protein used for biopanning, which was expressed in yeast, was structurally slightly different from the mature E2 glycoprotein on the CSFV virion. Nevertheless, the phage E2P4 displayed peptide is a potentially potent antiviral molecule worthy of further improvement and test. This study provides an alternative way to discover the substitutes for CSFV antibodies using the phage-display technology, which is possible to find the potential peptide mediating the interactions between CSFV and the host. Next we will try to map the epitope which the phage E2P4 binds to and modify the peptide to achieve an optimal antiviral activity. Another work will be focused on biopanning of landscape phages with high affinity and antiviral activity to the native E2 glycoprotein.

In summary, four phage clones specifically binding the E2 protein of CSFV were screened from f8/8 landscape phage library, and the phage E2P4 displaying octapeptide DRATSSNA significantly inhibited the CSFV infection in PK-15 cells at a higher titer.

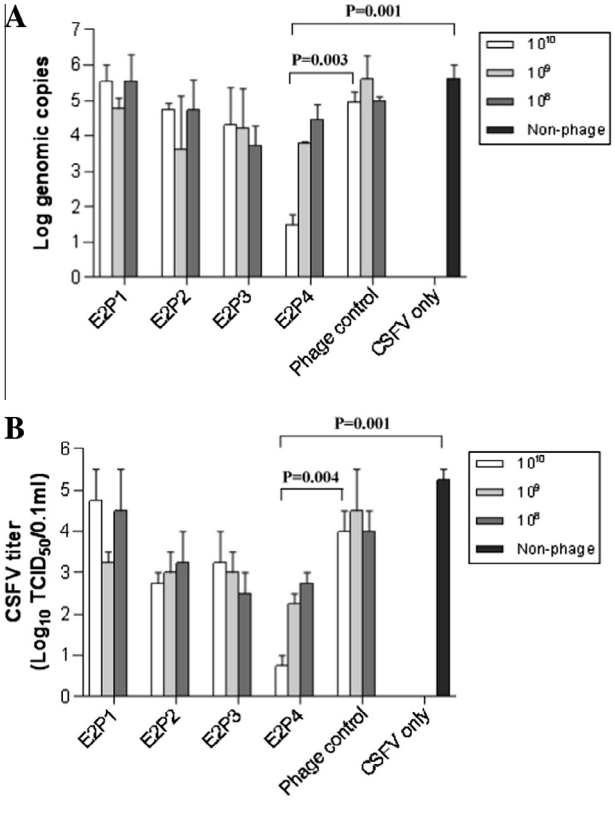

Fig. 3. Virus-blocking assay of phages that display E2-binding peptides. The phages that display E2-binding peptides and the irrelevant phage control (10^8–10^{10} TU) were incubated with 200 $TCID_{50}$ CSFV at 37 °C for 1 h. Then the virus that was treated with phages was inoculated onto PK-15 cells, and non-phage treated virus was used as a control. After 72-h incubation, the virus was harvested and quantitative real-time RT-PCR was carried out to determine the viral RNA copies (A). Meanwhile, the titers of infectious virus in PK-15 cell cultures were determined by titration based on immunofluorescence assay (B). Error bars represent standard deviation of three independent experiments.

Acknowledgements

This work was financially supported by Open Foundation of National Key Laboratory of Veterinary Biotechnology (No. SKLVBF201204), Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, National Natural Science Foundation of China (Nos. 91227116 and 21275152) and the Hundred-Talent-Project (No. KSCX2-YW-BR-7), Chinese Academy of Sciences (to A.L.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.06.012.

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