Journal of Clinical Virology 57 (2013) 305–310

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

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

Phages bearing affinity peptides to severe acute respiratory syndromes-associated coronavirus differentiate this virus from other viruses

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ARTICLE INFO

Article history:
Received 24 August 2012
Received in revised form 29 March 2013
Accepted 2 April 2013

Keywords:
SARS-CoV
Phage display
S protein
ELISA

ABSTRACT

Background: Transmission of SARS-associated coronavirus (SARS-CoV) is now well controlled, nevertheless, it is important to develop effective methods to identify this virus from other pathogens.
Objectives: The purpose of this study was to identify potential ligands and develop a novel diagnostic test to SARS-CoV using phage display technology.
Study design: The SARS-CoV spike 1 (S1) protein containing the receptor binding region (RBD) was used as an immobilized target followed by incubation with a 12-mer phage display random peptide library. After four rounds of biopanning, 10 monoclonal phages with specific binding activity to the S1-RBD protein were obtained and subjected to binding and diagnostic assays.
Results: DNA sequencing showed that two phage displayed peptides HHKTWHPPVMHL (phage-H) and SQWHPRSASYPM (phage-S) that were specific ligands to the S1 protein. Moreover, the selected phage-H and phage-S were capable of differentiating SARS-CoV from other coronaviruses in indirect enzyme-linked immunosorbent assays.
Conclusion: The peptides identified in this study are useful reagents for detection of SARS-CoV.

1. Background

Severe acute respiratory syndrome (SARS) caused by SARS-associated coronavirus (SARS-CoV) was a life-threatening disease and widespread in 2003. Although transmission of SARS-CoV has been well controlled currently, it is necessary to develop effective methods to identify this virus from other pathogens.

SARS-CoV is an enveloped, single-stranded positive-sense RNA virus with a genome that is about 30,000 nucleotides in length and encodes at least 15 open reading frames.[1,2] SARS-CoV encodes four major structural proteins: spike (S) protein, nucleocapsid (N) protein, membrane (M) protein and small envelope (E) protein.[1,2] Cell infection by SARS-CoV is initiated by the interaction of the surface S protein with human angiotensin-converting enzyme 2 (hACE2).[3–5] The hACE2 protein was identified as a functional receptor for the SARS-CoV and its binding site on the S protein was localized between amino acids 318 and 510.[3–5]

In a phage display peptide library, random peptides are expressed on the surface of a filamentous bacteriophage.[6–10] Phage library is a powerful molecular tool, allowing specific screening of optimal ligands of given targets based on an *in vitro* panning process. The phage display technology has been successfully applied for epitope selection,[11–13] drug discovery[9] and identification of ligands.[14–16]

2. Objectives

One purpose of this study was to identify potential ligands to SARS-CoV by the biopanning assay of SARS-CoV S1 protein with a phage display random library. Another purpose is to develop a phage-based ELISA to distinguish SARS-CoV from other control viruses.

3. Study design

3.1. Virus and other reagents

Transmissible gastroenteritis (TGEV) isolate HR/DN1, porcine epidemic diarrhea virus (PEDV) HLJBY,[17] porcine reproductive and

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http://dx.doi.org/10.1016/j.jcv.2013.04.002

respiratory syndrome virus (PRRSV) isolate HH08[18] and infectious bronchitis virus (IBV) strain HH06,[19] human coronavirus 229E (HCoV 229E)[20] and murine hepatitis virus strain A59 (MHV-A59)[21] were kept in our laboratory. The SARS-CoV strain BJ-01 was a generous gift from Dr. Chengfeng Qin, Beijing Institute of Microbiology and Epidemiology, China. For virus inactivation, the SARS-CoV was treated with β-propiolactone followed by concentration with PEG20000 and purification by Sepharose 4FF column chromatography as detailed in a previous report.[22] Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgG were purchased from the Zhongshan Company (Beijing, China).

3.2. Expression and purification of S1 protein in Escherichia coli

Expression of SARS-CoV S1 protein was performed as described previously.[23] A recombinant plasmid bearing full-length SARS-CoV S gene (GenBank accession number AY278554) was used as PCR template. Sense primer (CoVS1) 5′-GGGGggattcATGGGTTTTAACAC TTTG and antisense primer (CoVS2) 5′-CCCCgaattcCTTGTTGAAA TGGTTGAAA were synthesized to amplify a truncated S gene (nucleotides 664-1656). After inserting the S1 gene into BamHI and EcoRI sites of pGEX-6p-1 prokaryotic expression vector (Amersham Biosciences, New York, NY), the recombinant plasmid was sequenced and designed as pGEX-SARS-S1. The pGEX-SARS-S1 plasmid was transformed into host cells *E. coli* BL21(DE3). Expression of target protein was induced using 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C. Subsequent purification of inclusion bodies and renaturation of fusion protein by dialysis were performed as previously described.[23,24] The target protein was designated as S1 protein.

3.3. Biopanning and enrichment analysis

Phage display was performed using a Ph.D.-12 Phage Display Peptide Library Kit (E8110) according to the manufacturer's instructions (New England Biolabs) with minor modifications. For the first round of panning, 96-well plates were coated with the purified S1 protein at a concentration of 15 μg/well in 0.1 M $NaHCO_3$ (pH 8.6) buffer overnight at 4 °C. Then, these plates were blocked for 1 h at 4 °C with 5% skimmed milk diluted in 0.05% (v/v) Tween-20 in phosphate-buffered saline (PBST). Following six washes with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween-20), the S1 protein was incubated with the phage library at a final concentration of 1.5×10^{11} pfu (100 μL/well) at room temperature for 30 min with gentle rocking. The unbound phages were removed by 10-time washes with TBST and the bound phages were eluted by adding 100 μL elution buffer (0.2 M glycine–HCl [pH 2.2]) at room temperature for 30 min. The elute neutralized with 15 μL of 1 M Tris–HCl (pH 9.1) was harvested followed by amplification and titration in *E. coli* ER2738.

The second, third and fourth rounds of panning were done by similar panning processes with the exception of gradually increased concentration of Tween-20 (0.5%, v/v) in TBST. In-between each round of panning, the titer of the amplified phages in washing buffer (here referred to as Amplifying) and that in the elution buffer (here referred to as Output) was determined, and their ratio was analyzed to evaluate the enrichment efficiency.

3.4. Binding analysis of individual phage using ELISA

Ten phage clones were subjected to ELISA. Briefly, ELISA plates were coated with the S1 protein diluted in 0.1 M $NaHCO_3$ (pH 8.6) (10 μg/well) overnight at 4 °C. The mixed phages from the phage display library were used as a control. The second day, the plates were blocked with 1% TBSB for 2 h at room temperature. These plates were washed six times with TBST and then incubated with the selected monoclonal phage at a concentration of 1.5×10^{11} pfu/100 μL in 0.1 M $NaHCO_3$ (pH 8.6) for 1 h at 37 °C. After six washes with TBST, rabbit anti-M13 polyclonal antibody (diluted 1:1000 in TBSB; Abcam) was added to these wells for another 1 h at 37 °C. After six washes with TBST, these wells were incubated with HRP-conjugated anti-rabbit IgG antibody (diluted 1:1000 in TBSB). The color was developed using o-phenylenediamine (OPD), and the optical density (OD) value was read using an ELISA reader at a wavelength of 490 nm.

3.5. PCR amplifying genes encoding exogenous phage-displayed peptides

The ten positive phage clones were amplified and precipitated with polyethylene glycol–NaCl. Each phage clone DNA was purified using a plasmid extraction kit (Qiagen, Germany). The purified DNA template and the primers +130M13 (5′-TCACCTC GAAAGCAAGCTGA) and −28M13 (5′-CCCTCATAGTTAGCGTAACG) were used to PCR amplify the gene encoding the exogenous peptides of the M13 phages. DNA sequencing was carried out by the Borun Shanghai Company. The deduced amino acid sequences were analyzed using the Lasergene MegAlign software program (Lasergene 7, DNASTAR, Inc., USA).

3.6. Binding of phage-displayed peptide to transiently expressed SARS-CoV S1 protein

Binding of the identified phage-displayed peptides to the S1 protein was investigated. Recombinant plasmids bearing SARS-CoV S1 gene, TGEV S1 gene or PEDV S1 gene were constructed by inserting the respective gene into the pVAX-N1 vector resulting in pVAX-SARS-S1, pVAX-TGEV-S1 and pVAX-PEDV-S1, respectively. These plasmids were transfected into BHK 21 cells in 24-well plates using lipofectamine 2000™ transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. At 24 h post-transfection, indirect immunofluorescence assays were performed with modifications as described.[24,25] Cells fixed with 4% paraformaldehyde (w/v in PBS) were incubated with the identified phages and an unrelated phage bearing a control peptide (phage-control) (1.5×10^{11} pfu, diluted in PBS) for 1 h. After washing with PBS, the cells were incubated with the anti-M13 antibody (1:300 dilution in 1% BSA) followed by incubation with FITC-labeled goat anti-rabbit IgG (1:500 dilution in 1% BSA) for 1 h in the dark. After three washes with PBS, green fluorescence signals were analyzed by fluorescence microscope (Leica, Germany). The transient expression levels of the constructs were measured by selecting the central areas of the transfected cells (five wells/sample) to capture the fluorescence picture and the average fluorescence densities were determined with ImageJ software (developed at the National Institutes of Health).

3.7. Establishment of phage-mediated ELISA for virus diagnosis

The selected phages and phage library were used as diagnostic reagents to detect a panel of viruses composed of SARS-CoV, HCoV 229E, MHV-A59, TGEV, PEDV, PRRSV, and IBV; 0.1 M $NaHCO_3$ (pH 8.6) was used as negative control. All the viruses of the same titer (10^6 pfu/mL) were diluted in 0.1 M $NaHCO_3$ (pH 8.6) to a final concentration of 10 μg/well and coated onto ELISA plates overnight at 4 °C followed by ELISA analysis as above. The OD_{490} values were determined. At least three independent experiments were repeated. Statistical significance was evaluated using the *t*-test. The $p < 0.01$ was considered highly significant statistically.