Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHINESE JOURNAL OF BIOTECHNOLOGY
Volume 22, Issue 5, September 2006
Online English edition of the Chinese language journal

Cite this article as: Chin J Biotech, 2006, 22(5), 701−706.

RESEARCH PAPER

Identification of a Mimotope Peptide Bound to the SARS-CoV Spike Protein Specific Monoclonal Antibody 2C5 with Phage-displayed Peptide Library

HUA Rong-Hong, WU Dong-Lai, TONG Guang-Zhi*, WANG Yun-Feng, TIAN Zhi-Jun, ZHOU Yan-Jun

National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China

Abstract: This article aims to identify the epitope corresponding to SARS-CoV spike protein specific neutralizing monoclonal antibody (MAb) 2C5. The antibody was used as the target and three rounds of bio-panning were conducted with a phage-displayed peptide library. After the third panning, 20 phage-plaque clones were randomly picked and analyzed for the binding ability with the MAb 2C5 by ELISA. The displayed sequence analysis demonstrated that among the 20 phage clones, eight clones displayed the same seven-peptide TPEQQFT. All these eight phage-clones showed strongest binding activity with 2C5 in the phage ELISA analysis. Furthermore, phages displaying peptide TPEQQFT could specifically inhibit the binding of MAb 2C5 with SARS-CoV spike protein. The results demonstrated that TPEQQFT is a mimic epitope peptide containing neutralizing MAb 2C5. This study may provide information for further structural and functional analyses of spike protein and vaccine development for severe acute respiratory syndrome.

Key Words: severe acute respiratory syndrome coronavirus virus (SARS-CoV); spike protein; phage-displayed peptide library; mimotope

The spike (S) protein of SARS-CoV is a major virion structural protein. It plays an important role in cell tropism, specific binding with receptor, and cell-membrane fusion[1–4]. The spike protein is of good antigenicity and could induce neutralization antibodies. Among the structural proteins, the spike protein is the only significant neutralization antigen and protective antigen. Hence, it is a suitable candidate for genetic engineering of a subunit vaccine[5–8]. The development of SARS-CoV spike protein specific monoclonal antibodies (MAbs), especially that of neutralizing MAbs, are important in the prevention and diagnoses of SARS. Some research groups have reported MAbs of SARS-CoV[9–12]. In a structural and functional study of spike protein, identification of antigenic epitopes is important. Especially, identification of neutralizing epitopes may be beneficial for designing vaccine. To date, many linear antigenic epitopes are documented. Among these linear epitopes, some are neutralizing epitopes[13–16]. But on the spike protein, especially on the receptor-binding domain of the spike protein, most protective neutralizing epitopes are conformational epitopes[17]. Usually it is difficult to determine the specific sequence of discontinuous conformational epitopes. However, a phage-

Received: March 9, 2006; Accepted: April 27, 2006.
* Corresponding author. Tel/Fax: +86-451-82734181; E-mail: gztong@hvri.ac.cn
This work was supported by the Heilongjiang Postdoctoral Grant (No. HPD2003045).

displayed peptide library could be used to select the mimotope that could mimic the discontinuous or conformational epitopes. Here the phage-displayed peptide library was used to select the mimotope of a SARS-CoV spike protein specific neutralizing MAb 2C5. The results may provide information for further structural and functional research and for designing SARS vaccines.

1 Materials and methods

1.1 Phage-displayed peptide library and monoclonal antibody

The Ph.D.-C7C™ Phage-Displayed Peptide Library Kit was purchased from New England Biolabs Company. The library consists of 1.2×10^9 electroporated sequences. SARS-CoV spike protein specific neutralizing MAb 2C5 was provided by the Academy of Military Medical Sciences, China.

1.2 Bacterial strain, plasmids, and other materials

E. coli strain ER2378, Dh5α, and BL21 are all stored in our laboratory. Plasmids pGEX-6P-1 is also stored in our laboratory. The full-length SARS-CoV spike protein expressed with recombinant baculovirus was donated by Dr. Bu (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences). MAb against phage M13 was purchased from Promega.

1.3 Phage titering

Phages are titered according to the kit manual as follows: First, inoculate 5–10 mL of LB with a single clone of ER2738 and incubate with shaking until a mid-log phase (OD_{600}~0.5). When cells grow, melt agarose top in microwave and dispense 3 mL into sterile culture tubes, one per expected phage dilution. Equilibrate tubes at 50 °C until they are ready for use. Dilute phage in 10-fold serial dilution with LB. Once culture has reached the mid-log phase, dispense 200 μL of culture into microfuge tubes, one for each phage dilution. Add 10 μL of each dilution to each tube, vortex rapidly, and incubate at room temperature for 5 min. Transfer the infected cells, one at a time, to a culture tube containing 50 °C agarose top, vortex rapidly, and immediately pour onto a 37 °C pre-warmed LB/Tet/IPTG/X-Gal plate. Allow the plate to cool for 5 min, invert, and incubate overnight at 37 °C. Examine the plates and count plaques on the plate that contains about 100 plaques. Convert phage titers to plaque forming units (pfu) per 10 μL by multiplying each number by the dilution factor.

1.4 Panning procedure

Panning the peptide library against MAb 2C5 is carried out according to the kit manual. The main procedure is as follows: Microtiter plates are coated with MAb 2C5 in 0.1 mol/L $NaHCO_3$ (pH 8.6) at 4 °C overnight and are blocked at 4 °C for 2 h with blocking buffer. Wash plates five times with TBST. After washing, immediately follow panning and avoid drying out of the plates. Dilute 2×10^{11} phage with 100 μL TBST, pipette onto coated plates, and shake gently for 60 min at room temperature. Discard nonbinding phage by pouring off and tapping the plate face down onto a clean paper towel. Wash plates 10 times with TBST. Use a clean part of the paper towel each time to prevent cross-contamination. Elute bound phage with 100 μL elution buffer. Shake gently for 10 min at room temperature. The solution in the pipette was eluted into a microcentrifuge tube and neutralized with 15 μL 1mol/L Tris·HCl (pH 9.1). Titer 1 μL of the eluate as described above. Add the remaining eluate to 20 mL of ER2738 culture and incubate at 37 °C with vigorous shaking for 4.5 h. The amplified phage was used for the second round of panning. After three rounds of panning, titer the eluate and randomly take 20 plaques from the plates in which the total plaques are less than 100. These phage clones were amplified and designated 2C5P1, 2C5P2 to 2C5P20.

1.5 Phage sequencing

The randomly taken 20 phage clones were sequentially analyzed after amplification. The sequencing templates were rapidly purified as follows: After plaque amplification and first centrifugation, transfer 500 μL of the phage-containing supernatant to a fresh microfuge tube. Add 200 μL PEG/NaCl. Invert to mix, and allow it to stand at room temperature for 10 min. Centrifuge under 12 000 g for 10 min and discard supernatant. Suspend pellet thoroughly in 100 μL iodide buffer and add 250 μL ethanol. Incubate for 10 min at room temperature. Spin under 12 000 g for 10 min and discard supernatant. Wash pellet in 70 % ethanol, and dry for a short period of time in vacuum. Suspend pellet in 30 μL distilled water and use for sequencing templates. The sequencing primer is M13-96g11, and the sequence is 5′-GCCCTC ATAGTTAGCGTAGCT-3′. All phage sequencing works are completed by Invitrogen Co. Ltd (Shanghai, China).

1.6 Phage ELISA

Microtiter plates were coated with MAb 2C5 in 10 μg/mL, 100 μL per well at 4 °C overnight. After being washed thrice with TBST (TBS contains 0.5 %Tween-20), the plates were blocked with 5 mg/mL BSA in 37 °C for 1 h and then washed thrice. Dilute the purified phage into 10^{12} pfu/mL with TBS. Add 100 μL into one well, and for each sample, repeat the procedure for the three wells. Incubate at room temperature for 2 h and then wash five times. Add 100 μL 1:5 000 diluted HRP-conjugated mouse anti-phage M13 antibody to each well and incubate at 37 °C for 1 h, and wash five times. Color developed with the addition of *o*-phenylenediamine dihydrochloride and hydrogen peroxide. The reaction was stopped by the addition of 2 mol/L H_2SO_4 and the absorbance was measured at 490 nm by microplate autoreader (Bio-Rad).

1.7 Competitive ELISA

In competitive ELISA, the microtiter plates were coated with recombinant baculovirus-expressed spike protein. Before being incubated with coated microtiter plates, the primary antibody MAb 2C5 was first incubated with serial diluted phage 2C5EP14. The wild-type phage VcsM13 was used as

the control. HRP-conjugated goat antimouse IgG was used as the secondary antibody.

2 Results

2.1 Phage enrichment of bio-panning

To improve the efficiency and specificity of selection, the concentration of Tween-20 in washing buffer was increased during each round of panning. After three rounds of panning, the phage-displayed library resulted in an enrichment of phages that were bound to MAb 2C5. The output to input ratios of the three rounds of panning are shown in Table 1.

2.2 Selecting positive clones by phage ELISA

After the third round of panning, 20 phage clones were randomly taken. To analyze the binding ability of these phages to the MAb 2C5, phage ELISA was conducted. The results revealed that among the *OD* values of 20 phage clones, there are ten over 0.2, and eight over 0.25 (Fig. 1). The results demonstrated that after three rounds of panning, phage-displayed peptides that can bind MAb 2C5 were enriched.

Table 1 Bio-panning with the peptide library

	First screening	Second screening	Third screening
Phage input	2×10^{10} pfu	5×10^{8} pfu	5×10^{8} pfu
Phage output (elution)	1.7×10^{5} pfu	3.7×10^{5} pfu	1.9×10^{7} pfu
Phage out/Phage input	8.5×10^{-4} %	0.074 %	3.8 %

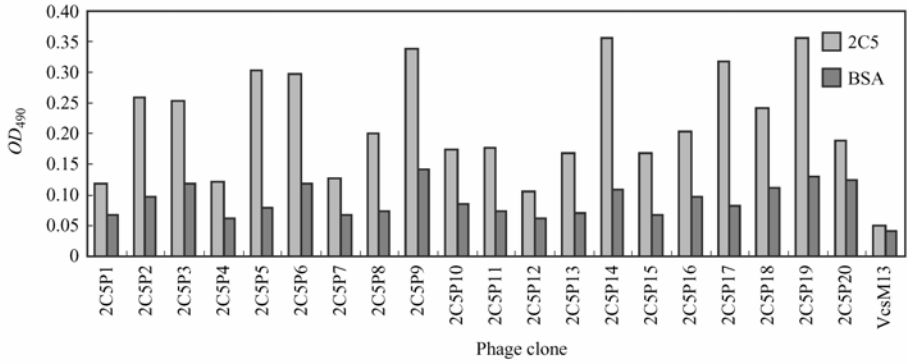

Fig. 1 ELISA analysis showing the binding ability of diluted phage clones to MAb 2C5

2.3 Analysis of the sequences of phage-displayed peptide

The sequencing results show that among the 20 randomly selected phage clones, eight clones displayed the same sequence peptide TPEQQFT. The phage ELISA *OD* values of these eight phage clones are over 0.25 (Table 2). The sequence analysis results were correct and coincided with those of phage ELISA. So the peptide TPEQQFT may mimic the epitope of MAb 2C5, and the peptide TPEQQFT is the mimotope of MAb 2C5. For further analysis of the epitope of MAb 2C5 on the spike protein, the peptide sequence was aligned with the spike protein sequence. And the alignment results showed that the peptide TPEQQFT may distribute in amino acid residues 539-559 (Fig. 2) on the spike protein of SARS-CoV. To confirm whether this region was the epitope of MAb 2C5 needs further experimental proof.

2.4 Phage competitive ELISA

Phage ELISA and sequence analysis results demonstrated that peptide TPEQQFT may be a mimotope of MAb 2C5. The results of phage competitive ELISA also verified the binding ability of phage clone 2C5P14 to MAb 2C5. The phage clone 2C5P14 displayed peptide TPEQQFT. When the amount of phage reached 10^{11} pfu per well, the binding of MAb with spike protein was nearly completely inhibited (Fig. 3).

```
Bj01   K N Q C V N F N F N G L T G T G V L T P S S K R F Q P F Q Q F G R D V S D F T D   560
2C5p-2 - - - - - - - - - - - - - - - - - - T P E - - - - - - - Q Q F - - - - - - - T     7

Bj01   K N Q C V N F N F N G L T G T G V L T P S S K R F Q P F Q Q F G R D V S D F T D   560
2C5p-2 - - - - - - - - - - - - - - - - - - T P E - - - - - - - Q Q - - - - - - - F T     7

Bj01   K N Q C V N F N F N G L T G T G V L T P S S K R F Q P F Q Q F G R D V S D F T D   560
2C5p-2 - - - - - - - - - - - - - - - - - - - - - - - - - - - - T P E Q Q F T - - - - - -     7
```

Fig. 2 Sequence alignment between the peptide displayed on the phage and the S protein of SARS-CoV of Bj01 strain

Table 2 Sequence analysis of selected clones

Phage clone No.	OD_{490}	DNA sequence	Amino acid sequence
2C5p1	0.119	ACGAAGTCTCCTCCTCTGCAG	TKSPPLQ
2C5p2	0.258	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p3	0.253	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p4	0.121	ACGAAGTCTCCTCCTCTGCAG	TKSPPLQ
2C5p5	0.302	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p6	0.298	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p7	0.127	ACGAAGTCTCCTCCTCTGCAG	TKSPPLQ
2C5p8	0.200	ACGAAGTCTCCTCCTCTGCAG	TKSPPLQ
2C5p9	0.338	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p10	0.173	ACTCCTTCGGCGCTTGCGAGT	TPSALAS
2C5p11	0.176	TCTTGGCCTAATACGAGTAAT	SWPNTSN
2C5p12	0.107	ACGTCGCTGCTGAGGGGGCAG	TSLLRGQ
2C5p13	0.168	CCTCCGATGCCGAATACGACG	PPMPNTT
2C5p14	0.357	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p15	0.167	ACTCCTTCGGCGCTTGCGAGT	TPSALAS
2C5p16	0.203	TCTTGGCCTAATACGAGTAAT	SWPNTSN
2C5p17	0.318	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p18	0.242	CATGCTACGCATACGAATTAT	HATHTNY
2C5p19	0.355	ACGCCTGAGCAGCAGTTTACT	TPEQQFT
2C5p20	0.187	ACTCCTTCGGCGCTTGCGAGT	TPSALAS

Eight clones (2, 3, 5, 6, 9, 14, 17, 19) OD_{490} values are over 0.25. And they all displayed the peptide TPEQQFT. Ten clones OD_{490} values are over 0.2, including eight clones displayed TPEQQFT, one displayed SWPNTSN (16), and one displayed HATHTNY (18).

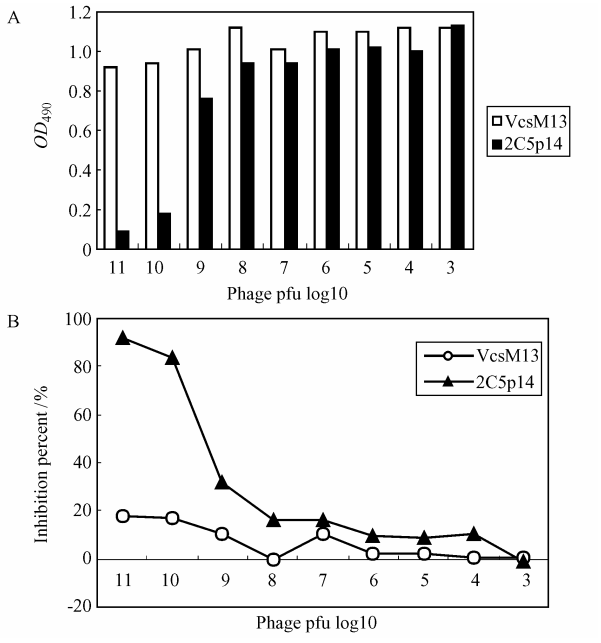

Fig. 3 Competitive inhibition ELISA of selected phage

3 Discussion

In 1985, Smith[18] first inserted foreign DNA fragments into the filamentous phage gene. The fusion protein was incorporated into the virion, which retains infectivity and displays the foreign amino acids in an immunologically accessible form. The foreign amino acids displayed on the phage surface could be purified by affinity chromatography to antibody directed against the gene product[19]. Peptides displayed on the surface of phage could retain its native structure and bioactivity. The fusion phage could be affinity-purified and selected. Therefore, it provided a powerful method for selected target proteins and peptides. Phage-display technique could be used to assess the interaction between proteins and peptides. And this technique now considerably benefits the research in designing new vaccine, and developing diagnostic reagents, antigenic epitope mapping, cell-surface engineering, and other affinity molecular purification[20–22].

Phage-display technique has been used to create a physical linkage between a vast library of random peptide sequences to the DNA encoding each sequence, allowing rapid identification of peptide ligands for a variety of target molecules by an *in vitro* selection process called panning. In this study, the MAb 2C5 was used as the selection target. The panning was carried out by incubating the Ph.D.-C7C™ Phage-Displayed Peptide Library with the microtiter plate coated with the target. After three rounds of panning, individual clones are characterized by DNA sequencing and phage ELISA. Among 20 clones, nearly 10 displayed peptide TPEQQFT. By aligning with the spike protein of SARS-CoV, the peptide TPEQQFT may distribute in amino-acid residues 539 to 559. But the fusion protein containing spike-protein fragment of amino-acid residues 539 to 559 could be

recognized by MAb 2C5. However, this fragment contains a linear epitope because this fusion protein could be recognized by SARS-CoV-immunized animal sera[14]. The phage-displayed peptide TRPQQFT could inhibit the binding between MAb 2C5 and the spike protein. So this peptide is the mimotope of MAb 2C5.

In Western blot analysis, MAb 2C5 could not recognize the reduced cell cultured SARS-CoV spike protein, recombinant spike protein, and recombinant spike protein fragments. So the epitope of MAb was not a linear epitope, and its epitope was dependent on its conformation. Recently, reports about the characteristics of MAb 2C5 also confirmed that the epitope of MAb 2C5 was a conformational epitope[23,24]. Monoclonal antibody 2C5 could neutralize SARS-CoV. It could inhibit the binding of spike protein receptor-binding domain with receptor ACE2. In this study, a peptide which could mimic the epitope of MAb 2C5 was identified. This result could provide useful information for further analyzing the function of spike protein and for the development of effective SARS vaccine.

REFERENCES

[1] Babcock GJ, Esshaki DJ, Thomas WD Jr, *et al.* Amino acids 270 to 510 of the severe acute respiratory syndrome coronavirus spike protein are required for interaction with receptor. J Virol, 2004, 78(9): 4552–4560.

[2] Wong SK, Li W, Moore MJ, *et al.* A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. J Biol Chem, 2004, 279(5): 3197–3201.

[3] Gallagher TM, Buchmeier MJ. Coronavirus spike proteins in viral entry and pathogenesis. Virology, 2001, 279(2): 371–374.

[4] Hofmann H, Pohlmann S. Cellular entry of the SARS coronavirus. Trends Microbiol, 2004, 12(10): 466–472.

[5] Bisht H, Roberts A, Vogel L, *et al.* Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. PNAS, 2004, 101(17): 6641–6646.

[6] Buchholz UJ, Bukreyev A, Yang L, *et al.* Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. PNAS, 2004, 101(26): 9804–9809.

[7] Bukreyev A, Lamirande EW, Buchholz UJ, *et al.* Mucosal immunization of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet, 2004, 363(9427): 2122–2127.

[8] He Y, Zhou Y, Liu S, *et al.* Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. Biochem Biophys Res Commun, 2004, 324(2): 773–781.

[9] Berry JD, Jones S, Drebot MA, *et al.* Development and characterization of neutralizing monoclonal antibody to the SARS-coronavirus. J Virol Methods, 2004, 120(1): 87–96.

[10] Greenough TC, Babcock GJ, Roberts A, *et al.* Development and characterization of a severe acute respiratory syndrome-associated coronavirus-neutralizing human monoclonal antibody that provides effective immunoprophylaxis in mice. J Infect Dis, 2005, 191(4): 507–514.

[11] Zhou YJ, Hua RH, Wang YF, *et al*. Development of monoclonal antibodies against SARS-CoV and identification of antigenic epitopes. Chinese Journal of Biotechnology, 2005, 21(2): 211–215.

[12] Gubbins MJ, Plummer FA, Yuan XY, *et al.* Molecular characterization of a panel of murine monoclonal antibodies specific for the SARS-coronavirus. Mol Immunol, 2005, 42(1): 125–136.

[13] Hua RH, Tong GZ, Wang YF, *et al*. Expression and antigenic epitopes mapping of receptor binding domain on the spike protein of severe acute respiratory syndrome coronavirus. Prog Biochem Biophys, 2005, 32(11): 1030–1037.

[14] Hua RH, Wang YF, Bu ZG, *et al*. Identification and epitope mapping of an immunodominant region on S1 domain of SARS-CoV spike protein. DNA and Cell Biology, 2005, 24(8): 503–509.

[15] Hua RH, Zhou YJ, Wang YF, *et al*. Identification of two antigenic epitopes on SARS-CoV spike protein. Biochem Biophys Res Commun, 2004, 319(3): 929–935.

[16] Zhang H, Wang G, Li J, *et al.* Identification of an antigenic determinant on the S2 domain of the severe acute respiratory syndrome xoronavirus spike glycoprotein capable of inducing neutralizing antibodies. J Virol, 2004, 78(13): 6938–6945.

[17] He Y, Lu H, Siddiqui P, *et al.* Receptor-binding domain of severe acute respiratory syndrome coronavirus spike protein contains multiple conformation-dependent epitopes that induce highly potent neutralizing antibodies. J Immunol, 2005, 174(8): 4908–4915.

[18] Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science, 1985, 228(4705): 1315–1317.

[19] Parmley SF, Smith GP. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene, 1988, 73(2): 305–318.

[20] Hou L, Du G, Tong Y, *et al.* Identification of B cell epitopes of hepatitis C virus RNA dependent RNA polymerase. J Virol Methods, 2002, 104(1): 1–8.

[21] Dell'Orco M, Saldarelli P, Minafra A, *et al.* Epitope mapping of Grapevine virus A capsid protein. Arch Virol, 2002, 147(3): 627–634.

[22] Cerino A, Meola A, Segagni L, *et al.* Monoclonal antibodies with broad specificity for hepatitis C virus hypervariable region 1 variants can recognize viral particles. J Immunol, 2001, 167(7): 3878–3886.

[23] He Y, Zhu Q, Liu S, *et al.* Identification of a critical neutralization determinant of severe acute respiratory syndrome (SARS)-associated coronavirus: importance for designing

SARS vaccines. Virology, 2005, 334(1): 74–82.

[24] Chou TH, Wang S, Sakhatskyy PV, *et al*. Epitope mapping and biological function analysis of antibodies produced by immunization of mice with an inactivated Chinese isolate of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). Virology, 2005, 334(1): 134–143.