ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(3): 205–209 **CN** 31-1940/Q

Application of Phage-displayed Single Chain Antibodies in Western Blot

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Abstract A phage display single chain fragment variable library constructed on pIII protein of M13 filamentous phage was screened using B-lymphocyte stimulator and FP248 as selective molecules. After four rounds of panning, there was a remarkable enrichment in the titer of bound phages. Twenty phage clones were selected from the last round and screened by means of phage-ELISA. With the antibody phages as primary antibodies in Western blot, we developed a method for detecting the specific antigen. The dilutions of antibody phages depend on the affinity between antibody-displayed phage particles and antigens.

Key words phage-display; antibody; Western blot

The first surface expression system was developed by George P. SMITH in the mid-1980s, who displayed the peptides and small proteins fused with the pIII protein of the filamentous phage on the surface of bacteriophage [1]. In recent years, the filamentous bacteriophages have been used extensively for the display of large repertoires of antibodies on their surface. Phage-display enables rapid selection of antibodies from a large single chain fragment variable (scFv) library by virtue of the binding to certain target antigen. After two or three rounds of selection, the resulting phage population is markedly enriched for the scFvs that bind to the antigen [2]. Antibodies against hundreds of target antigens have so far been obtained from phage display antibody libraries, including cell-surface markers [3], peptide hormones [4], other human proteins [5] and carbohydrates [6].

One of the prominent advantages of the phage-display technology is that, once a library has been created, it can be used to select antibodies that bind to any target antigens of interest. A single phage-antibody library can be distributed to thousands of users and serve as the source of cloned antibodies against an unlimited array of antigens. This technology is also efficient, and the process of selection and primary screening is very rapid and can be completed within 2–3 weeks [7,8]. The selected phage clones are then subjected to immunodetection to confirm their binding activities. Enzyme-linked immunosorbent assay (ELISA) and Western blot are extensively used in immunodetection. Conventionally, the procedure usually involves subcloning and expression of the target fragments encoding the scFvs to obtain significant quantities of antibodies, which proves to be time-consuming and laborious [8,9]. To resolve this problem, researchers have recently focused on the use of selected phage clones in immunodetection or function studies. The specificity of selected phages has been extensively assessed with immobilized proteins and cells by using phage-ELISA [10,11]. For example, Al-Bukhari *et al*. [12] successfully blotted the phage clones onto nitrocellulose membranes as immobilized antigens in Western blot analysis.

B-lymphocyte stimulator (BLyS) is a newly identified member of the tumor necrosis family (TNF) superfamily and plays a key role in stimulating B-cell proliferation, differentiation and survival [13]. Antagonists of BLyS therefore have potentially important therapeutic value in the treatment of B-cell-associated autoimmune disease. FP248 is a protein associated with liver cancer.

In this study, a phage-display scFv antibody library constructed on the N terminus of pIII protein of M13

Received: November 23, 2004 Accepted: January 7, 2005

Abbreviations: BLyS, B-lymphocyte stimulator; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IPTG, isopropyl β-*D*-thiogalactopyranoside; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.5% Tween-20; PEG, polyethylene glycol; pfu, plaque forming unit; PVDF, polyvinylidene difluoride; scFv, single-chain fragment of variation; TMB, tetramethylbenzidine

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filamentous phage was screened by BLyS and FP248. The selected positive phages against these two proteins were successfully applied to Western blot as primary antibodies, which obviates the need for subsequent expression of the corresponding antibody, providing a simple and effective method of producing primary antibodies for Western blot.

Materials and Methods

Materials

Immunotubes were purchased from Nunc (USA). Horseradish peroxidase (HRP)-conjugated mouse anti-M13 antibody was obtained from Amersham Pharmacia (USA). Protein FP248 was provided by Prof. Jianren GU (Shanghai Cancer Institute, Shanghai, China). BLyS was prepared as previously described [14]. The vector pFUSE5, helper phage VCSM 13 and XL1-Blue bacteria were stored in our laboratory. All other chemicals were of analytical grade and commercially available.

Phage display library

RNA was isolated from the murine spleen immunized with purified BLyS. First-strand cDNA was synthesized by using cDNA synthesis kit (Amersham Pharmacia). The resulting cDNA was used as the template for PCR amplification of variable heavy (V_H) and variable light (V_L) chain gene fragments. The coding sequences of V_H and V_L of the antibody were linked by a 15 amino acids linker sequence to form *scFv* gene. The *scFv* genes were digested with *Sfi*I, agarose gel-purified, and ligated into the phage-display vector pFUSE5 that had been digested with the same restriction enzyme. The ligated products were electroporated into *Escherichia coli* XL1-Blue cells. The cells were plated on super broth (SB) medium (containing 1% glucose, 50 μg/ml ampicillin, and 40 μg/ml tetracycline) and incubated overnight at 37 °C [15].

Biopanning

The phage library was subjected to four rounds of biopanning. Immunotubes were coated with 1 ml BLyS or FP248 (5 μg/ml) in PBS overnight at 4 °C. After blocking with 5 ml of 4% skimmed milk for 1 h at 37 °C, 1 ml of 1.0×10^{11} pfu phages were added and incubated for 2 h at 37 °C. The tubes were washed 20 times with distilled water followed by washing with phosphate-buffered saline (PBS) and PBS containing 0.5% Tween-20 (PBST) in turn (15 times for round 1, 20 times for round 2, 3 and 4). Each time, the tubes were incubated for 1 min at room temperature. The bound phage clones were eluted with 1 ml of 0.2 M glycine-HCl (pH 2.2) for 10 min at 37 °C, and then neutralized with 60 μl of 2 M Tris. All the eluted phages were incubated with exponentially growing culture of *E. coli* XL1-Blue (3 ml) for 20 min at room temperature. The infected XL1-Blue cells were plated on the pre-warmed SB medium and incubated overnight at 37 °C.

Rescue of scFv-displaying phages

To rescue scFv-displaying phages, the clones were scraped off the plates and collected into 50 ml SB medium containing 1% glucose, 50 μg/ml ampicillin, and 10 μg/ml tetracycline. The culture was then shaken at 37 °C for 2.5–3.5 h, and 0.5 ml VCSM13 helper phages (10^{11} pfu) and 80 μl of 1 M IPTG were added. After 30 min incubation at room temperature, the culture was diluted with 50 ml SB and grown at 30 °C. After 2 h, 70 μg/ml kanamycin was added and the culture was allowed to grow overnight. After being centrifuged at 6000 rpm for 20 min, the supernatant was collected and added with 4% (*W*/*V*) PEG8000 and 3% (*W*/*V*) NaCl. The mixture was precipitated on ice for 30 min and centrifuged at 12,000 rpm for 20 min. The precipitated phages were collected, and resuspended in PBS. The phages were re-precipitated and the titer of the phages was assayed [16].

ELISA to identify positive phage clones

The 96-well plates were coated with 5 μg/ml BLyS or FP248 in PBS overnight at 4 °C and blocked with 300 μl 4% skimmed milk. The phages (1×10^{10} pfu) were added and incubated for 1 h at 37 °C. After washing with 0.05% Tween-20/TBS six times, the bound phage clones were detected with HRP-conjugated mouse anti-M13 antibody (1:1000). For detection, tetramethylbenzidine (TMB) substrate was used and the reaction was stopped with 2 M H_2SO_4. A_{450} was determined by using a microplate reader. In the negative control group, the phage clones were replaced by the VCSM13 helper phages.

Affinity test

The prepared phages were 10-fold serially diluted in SB from 10^6 to 10^{10} pfu. ELISA was carried out as described above.

Phage-Western blot

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed as routine methods. Briefly BLyS and FP248 were separated by 15% SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with

antibody phages (10^{10}, 10^9 pfu) in 10 ml of 4% skimmed milk for 1 h at room temperature. The bound phages were detected with HRP-conjugated mouse anti-M13 antibody (1:1000).

Results

Biopanning

A phage-display scFv antibody library, derived from murine and fused on the N terminus of pIII protein of M13 filamentous phage, was screened by protein BLyS and FP248, respectively. The library was highly variable and contained approximately 10^6 different clones of scFv antibody. The panning procedure was performed for four rounds totally, and the ratio of loaded versus eluted phages for each round is listed in **Table 1**. The recovery ratio of the last round was about 10^4 for BLyS and 10^2 times for FP248 respectively, which was higher than that of the first round. It indicated that the binding phages for the target antigens were enriched after biopanning.

Selection of phage clones

Twenty clones were selected randomly from the fourth round and screened by phage-ELISA to identify those that could strongly bind to the target proteins. Most of them showed positive signals. With this assay, clone B16 to BLyS and F1 to FP248 showed the highest absorbance at wavelength 450 nm. The results are shown in **Fig. 1**.

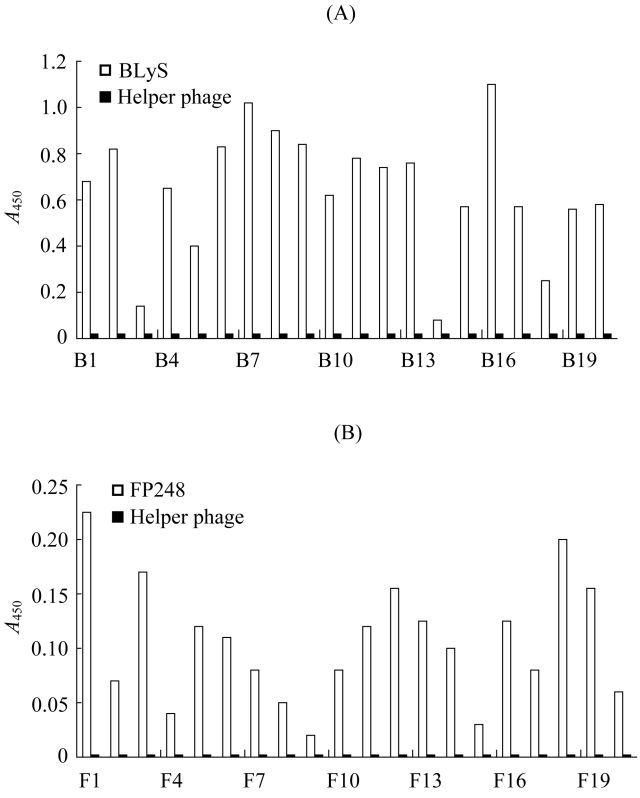

Fig. 1 ELISA to detect the interaction of protein with phage clones from antibody library

The ELISA was performed as described above. (A) Binding activity between individual phage clones and B-lymphocyte stimulator (BLyS). (B) Binding activity between individual phage clones and FP248.

The binding affinity of B16 to BLyS and F1 to FP248

To further analyze the binding activity of phage clone B16 to BLyS and F1 to FP248, a single plaque of the phage clones was amplified in 200 ml SB and precipitated by PEG/NaCl. Resuspended in PBS buffer, 10-fold serial dilutions of the phage supernatant were tested by phage-ELISA. As shown in **Fig. 2**, the affinity of clone B16 to BLyS was much stronger than clone F1 to FP248.

Phage-Western blot

To determine whether the phage-displayed scFv antibodies could be used directly as primary antibodies in Western blot, two antibody phages were applied to incubate with their corresponding antigens. The results are shown

Table 1 Phage recovery during screening phage display library

Round	Loaded phages (pfu)	Eluted phages (pfu)		Phage recovery (%)	
		BLyS	FP248	BLyS	FP248
1	1.0×10^{11}	9.0×10^{3}	3.4×10^{4}	9.0×10^{-6}	3.4×10^{-5}
2	1.0×10^{11}	1.7×10^{5}	6.7×10^{4}	1.7×10^{-4}	6.7×10^{-5}
3	1.0×10^{11}	3.7×10^{6}	5.0×10^{5}	3.7×10^{-3}	5.0×10^{-4}
4	1.0×10^{11}	1.4×10^{7}	4.5×10^{6}	1.4×10^{-2}	4.5×10^{-3}

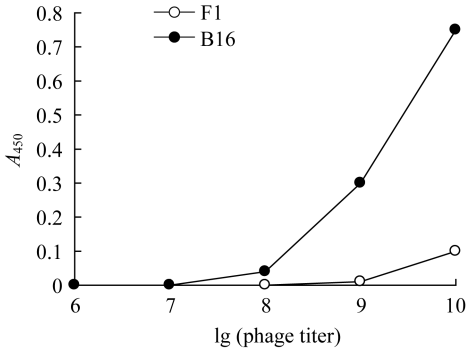

Fig. 2 Affinity of B16 to B-lymphocyte stimulator (BLyS) and F1 to FP248

in **Fig. 3**.

The serially diluted positive antibody phages, B16 or F1, were used as primary antibodies directly in Western blot, with helper phage and bovine serum albumin (BSA) as negative controls and immunized rabbit or mouse serum as positive control. When 10^{10} pfu of phages were applied, both B16 and F1 could identify the target proteins, including the purified and unpurified proteins. With 10^9 pfu of phages, the FP248 protein on the PVDF membrane could not be detected efficiently. The detection limit for BLyS seems to be 10^8 pfu phages, for the protein can not be detected at this titer of phages. Combined with binding assays, the results imply that the affinity of antibody phages to the target protein might affect the sensitivity of phage-Western blot assays. That is, phages with higher affinity to target proteins are more sensitive than lower ones. For the negative controls, no interaction was detected.

The results above indicate that phage display scFv antibodies could be used like monoclonal or polyclonal antibodies in Western blot successfully and specifically. Additionally, antibody phages could also be produced with a relatively simple and economical approach, suggesting that antibody phages would be promising in immunodetection or functional analysis of target proteins.

Discussion

Western blot is a popular method of detecting one protein in a mixture of any number of proteins and providing information about the size of the protein. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein. Primary antibody in Western blot is usually polyclonal antibody or monoclonal antibody produced by the hybridoma technique. In the current study, we developed a useful method to provide specific primary antibody for Western blot. First, a phage display scFv antibody library constructed on pIII was screened by biopanning using BLyS and FP248 as selective molecules. After four rounds of biopanning, phage clones with high affinity were enriched for the two proteins. Then a total of 20 phage clones were randomly selected from the last round and were screened by means of phage-ELISA. Lastly, we

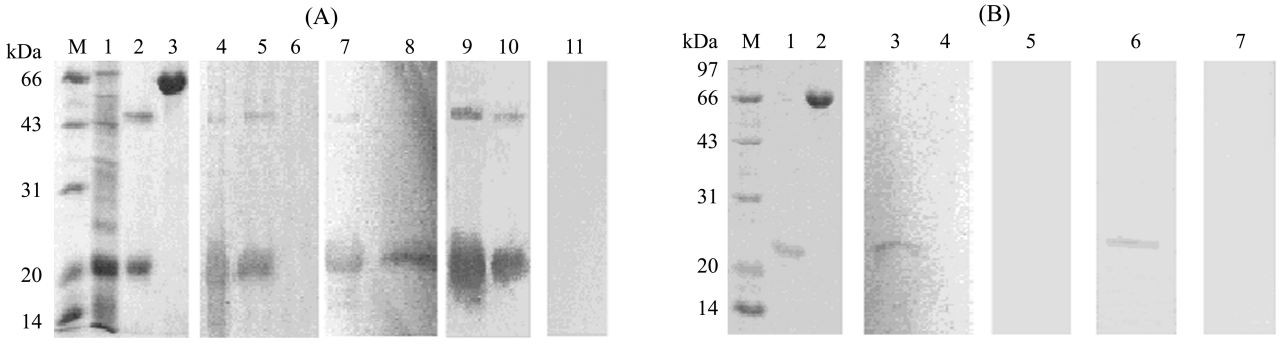

Fig. 3 Phage-Western blot

The target proteins (purified or unpurified) or positive and negative controls were separated by SDS-PAGE and transferred to PVDF membrane as described in "Materials and Methods". The antibody phage B16 or F1 was diluted serially then used as the primary antibody to detect the separated proteins. (A) Phage-Western blot results of B-lymphocyte stimulator (BLyS). M, marker; 1, SDS-PAGE of unpurified BLyS; 2, SDS-PAGE of purified BLyS; 3, SDS-PAGE of BSA; 4, 5 and 6, phage-Western (with 10^{10} pfu phages) of unpurified BLyS, purified BLyS and BSA; 7 and 8, phage-Western (with 10^9 pfu phages) of unpurified BLyS and purified BLyS; 9 and 10, Western of unpurified and purified BLyS with immunized rabbit serum (1:1000); 11, phage-Western of BLyS with 10^{11} pfu helper phages. (B) Phage-Western blot results of FP248. M, marker; 1, SDS-PAGE of purified FP248; 2, SDS-PAGE of BSA; 3 and 4, phage-Western (with 10^{10} pfu phages) of purified FP248 and BSA; 5, phage-Western of purified FP248 with 10^9 pfu phages; 6, Western of purified FP248 with immunized mouse serum (1:1000); 7, phage-Western of purified FP248 with 10^{11} pfu helper phages.

described the approach to detect the antigens with antibody phages as primary antibodies in Western blot directly, and the dilutions of the phage clones depended on the affinity between phages and antigens.

As the results showed, the selected phages could identify both the purified and unpurified proteins, and did not bind to BSA, the negative control. This indicates that the antibodies displayed on the phages could bind specifically to the purified or unpurified target proteins. The other control, the helper phages, were also used and no interactions were detected. All these results prove that phage clones could act as antibodies directly and specifically in Western blot.

In brief, the use of the phage-displayed monoclonal antibodies in Western blot offers an efficient and simple method of detecting specific antigens. There is no need to prepare large amounts of antigen, and the method avoids the time-consuming procedures of immunization and hybridoma technology, bypassing the immune system and immunization procedure. From this point of view, the method using the select antibody phages themselves as the probe in Western blot we described here is more useful.

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