Research paper

A fast and efficient procedure to produce scFvs specific for large macromolecular complexes

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Abstract

We have expanded the application of antibody phage display to a new type of antigen: ribonucleoprotein (RNP) complexes. We describe a simple and efficient method for screening antibodies specific for large intact RNPs and individual components. We also describe a fast and easy method to overcome the abundance of amber stop codons in the positive phage clones. The resulting antibodies have been used in ELISA and Western blot analysis.
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1. Introduction

Antibody phage display is an attractive and powerful alternative to hybridoma technology. Beyond generating antibodies against a wide variety of proteins (Dong et al., 2003; Smith et al., 2003; Chang et al., 2006; Marcus et al., 2006), it has also been used to produce antibodies specific for unconventional antigens includ-

ing small peptides (Rodriguez-Diaz et al., 2004), DNA photoproducts (Zavala et al., 2000), snRNAs (Teunissen et al., 1998) and lipids (Takkinen et al., 1996). However, this technology has not been efficient in producing antibodies that target subunits of macromolecular complexes (Rubinstein et al., 2003). Here we describe an optimized protocol to generate multiple monoclonal scFvs against a large ribonucleoprotein, the *E. coli* signal recognition particle–receptor complex.

Signal recognition particle (SRP) and its receptor (SR) are conserved in all organisms (Pool, 2005) as part of the molecular machinery that guides integral membrane and secretory proteins to the cellular translocation apparatus during translation (for details refer to Pool, 2005). *E. coli* SRP has two components: a protein subunit called Ffh (Bernstein et al., 1989; Romisch et al., 1989) and an 114-nucleotide RNA called 4.5S RNA (Poritz et al., 1990). *E. coli* SR is a single polypeptide molecule called FtsY (Luirink et al., 1994;

Abbreviations: scFv, single chain variable fragment; RNP, ribonucleoprotein; SRP, signal recognition particle; SR, signal recognition particle receptor; ELISA, enzyme linked immunosorbent assay; DTT, dithiothreitol; GMPPCP, guanylyl 5′-(beta, gamma-methylenediphosphonate); RT, room temperature; PBST, phosphate buffered saline with Tween-20; TBS, tris buffered saline; HRP, horse radish peroxidase; TMB, 3,3′5,5′-tetramethylbenzidine; AP, alkaline phosphatase; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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Miller et al., 1994). Ffh and FtsY each contain a homologous NG domain (Freymann et al., 1997; Montoya et al., 1997) which has GTPase activity and these domains associate to form a heterodimer upon GTP binding. Ffh contains an additional domain, the M domain, which binds to 4.5S RNA, whereas FtsY includes an additional domain – the A domain – which

helps anchor FtsY to the membrane (de Leeuw et al., 1997). A three-dimensional model of SRP-SR predicts that the 4.5S RNA lies at the interface of Ffh and FtsY in the complex (Spanggord et al., 2005) (Fig. 1A). In this report we optimized the phage display protocol and generated scFv antibodies specific for the intact SRP–SR RNP complex or its components.
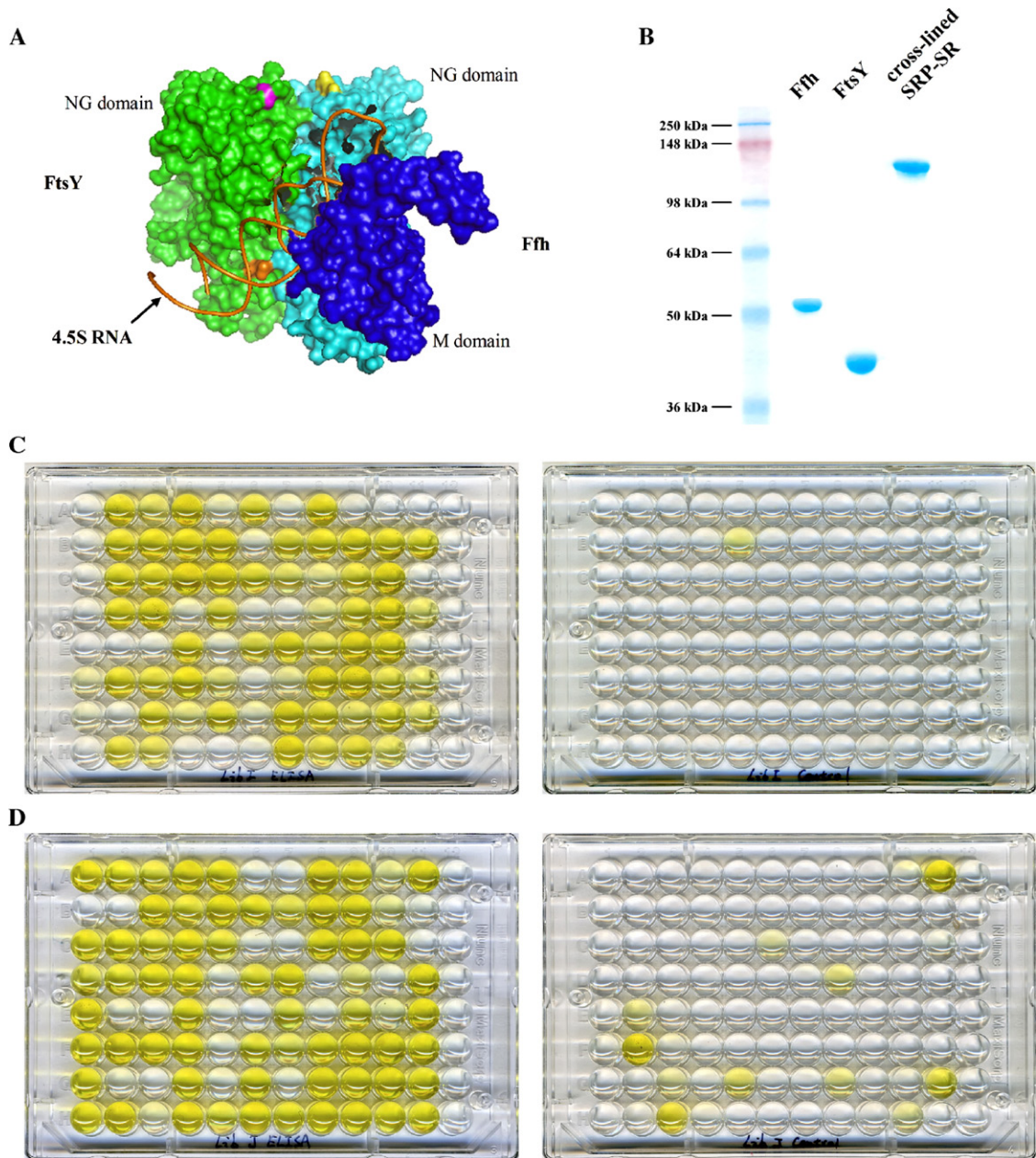

Fig. 1. Panning for scFvs specific for SRP–SR. (A) 3D model of SRP–SR complex showing the spatial relations of Ffh, FtsY and 4.5S RNA. (B) SDS-PAGE analysis of cross-linked SRP–SR. (C) Monoclonal ELISA plate of Tomlinson I library (left) and its control (right). (D) Monoclonal ELISA plate of Tomlinson J library (left) and its control (right).

2. Materials and methods

2.1. Preparation of cross-linked SRP–SR complex

Equal molar amounts of Ffh and FtsY were mixed at a final concentration of 20 µM in binding buffer (20 mM HEPES pH7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT and 2.5 mM GMPPCP) and incubated at room temperature (RT) overnight. The next day, the same molar amount of 4.5S RNA was heated at 80 °C for 8 min, cooled on ice for 10 min, and added to the Ffh–FtsY binding mixture. After a one-hour incubation at 37 °C, glutaraldehyde was added to a final concentration of 0.01%. After incubating at RT for 30 min, Tris pH 7.5 was added to a final concentration of 0.1 M to quench the reaction. The cross-linked SRP–SR complex was purified on a MonoQ column (GE Healthcare), concentrated to 10 µM and stored at −80 °C.

2.2. Improved method to select SRP-SR specific phage clones

Human single-fold scFv libraries Tomlinson I+J were obtained from the Medical Research Council (Cambridge, UK) which came with E. coli strains TG1 (for phage amplification), HB2151 (for scFv production), KM13 helper phage and one copy of a protocol (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf).

All incubations were performed at RT unless specified. Immunotubes (Nalge Nunc International USA) were coated with 100 pmol of cross-linked SRP–SR complex for 2 h, then blocked with blocking reagents (see below) at 4 °C overnight. 10^{13} phage were mixed with the blocking reagent (see below) and added to the tubes. After incubating for 2 h, the tubes were washed 20 times with PBST (see below), and bound phage were eluted with 1 mg/ml trypsin. E. coli TG1 was infected with eluted phage and KM13 helper phage to amplify phage for the next round of panning. Four rounds of panning were carried out in total.

To reduce the number of false positive phage clones, we devised the following modifications to the protocol. First, we added a pre-absorption step at each round. The phage solution was incubated with a blocked tube (no antigen) for 30 min then transferred to the antigen coated and blocked tube. We also used immunotubes with two kinds of surface materials: maxisorp and polysorp, and prepared two blocking reagents: 3% BSA in PBS and SuperBlock® in TBS (Pierce). Alternative pairs of tubes and blocking reagents were used between consecutive rounds of panning. For example, if maxisorp tubes and BSA were used in the first round, polysorp tubes and SuperBlock® were used in the second round. The third modification we introduced was to increase the concentration of Tween-20 in PBST by 0.05% in each round of panning. For example, we used 0.1% Tween-20 in the first round, 0.15% in the second round, 0.2% in the third round, and so on.

2.3. Monoclonal phage ELISA

Monoclonal phage ELISA was performed according to the provided protocol. Briefly, 88 clones from each of the two libraries were inoculated into column 1 through column 11 of 96-well plates, and KM13 helper phage were added to amplify phage particles. Additional plates (ELISA plates) were coated with 10 pmol cross-linked SRP–SR per well and blocked with 3% BSA in PBS. Phage-containing supernatants were transferred from the phage-producing plates to the corresponding wells of the ELISA plates. Column 12 of the ELISA plates showed the background signal because no phage was applied to it. Bound phage were detected by HRP/Anti-M13 monoclonal conjugate (GE Healthcare) (1/2000) and 1-Step™ Ultra TMB-ELISA (Pierce). Parallel ELISA assays using blocked plates (no antigen) were performed to identify any false positive clones.

2.4. Sequence analysis

Double strand phagemid DNAs were extracted from TG1 culture expanded from each positive wells of monoclonal phage ELISA plates, and sequenced with LMB3 (5′ CAGGAAACAGCTATGAC 3′) and pHEN seq (5′ CTATGCGGCCCCATTCA 3′) primers.

2.5. Expression and purification of scFvs

To solve the difficulties posed by extra amber condons in positive phage clones, scFv coding sequences were cut out of pIT2 phagemid with Nco I and Not I (New England Biolabs) and inserted into pET26b vector (EMD Biosciences) cut with the same enzymes. The pET26b–scFv plasmids were then transformed into RosettaBlue(DE3) (EMD Biosciences), which is an amber suppressor strain. Transformed RosettaBlue(DE3) cells were shaken at 37 °C in M9 media with 50 µg/ml kanamycin, 12.5 µg/ml tetracycline and 34 µg/ml chloramphenicol until O.D.$_{600}$ reached 0.6, then induced with 1 mM IPTG at 30 °C for 16 h. Harvested cells were subjected to osmotic shock to produce periplasmic extract.

For every liter of induced culture, harvested cells were resuspended in 20 ml ice cold TES (0.2 M Tris pH8.0, 0.5 M EDTA pH 8.0, 0.5 M sucrose), then mixed with 33 ml ice cold 20% TES, and incubated on ice for 30 min. The spheroplasts and the periplasmic fraction were separated by centrifugation. Active soluble scFvs enriched in the periplasmic fraction were first purified on Ni-NTA agarose (QIAGEN) columns, then further purified on ImmunoPure® immobilized protein L (Pierce) columns. The purified scFvs were concentrated to 100 µg/ml and stored at −80 °C.

2.6. ELISA with scFvs

The layout of the ELISA is shown in Table 1. 10 pmol per well of the following targets were coated on 96-well plates: cross-linked SRP–SR, Ffh, NG domain of Ffh (hNG), M domain of Ffh (hM), FtsY, NG domain of FtsY (yNG) and 4.5S RNA. The plates were then blocked with 3% BSA. Purified scFvs (100 µg/ml) were diluted 1/100 in 3% BSA before application to ELISA plates. ImmunoPure® protein L-HRP (Pierce) (1/1000) was used to detect bound scFvs. HRP substrate TMB was then added, the reaction was stopped by 1 M H_2SO_4. The absorbance at 450 nm of each well was measured with Spectra MAX 190 plate reader (Molecular Devices).

2.7. Western blots and dot blots with scFvs

10 pmol of SRP–SR (not cross-linked) complex was either run in each lane of SDS-PAGE and transferred to PROTRAN BA 83 nitrocellulose membrane (Whatman Schleicher and Schuell), or spotted directly on the same type of membrane. The membranes were blocked with SuperBlock® (Pierce). Each lane/dot on the membrane was cut off and incubated with purified scFv diluted 1/

100 in SuperBlock®. ImmunoPure® protein L-HRP (Pierce) (1/1000) was used to detect bound scFvs. For the positive control lane/dot, mouse anti-SRP–SR serum (1/2000) was used in place of scFv, and Goat anti-mouse IgG-AP (Santa Cruz Biotechnology) (1/5000) was used to detect bound antibodies.

3. Results and discussion

3.1. Preparing cross-linked SRP–SR complex

To ensure that the SRP–SR complex maintained its ternary structure during the panning process, it was cross-linked before bound to the tubes. Purified cross-linked SRP–SR was analyzed in SDS-PAGE side by side with purified Ffh and FtsY (Fig. 1B). The strong single band indicates that the cross-linking and purification procedures worked very well. The presence of 4.5S RNA was also confirmed by running the cross-linked complex in a non-denaturing gel and staining with both ethidium bromide and GelCode® Blue Stain Reagent (Pierce) (data not shown).

3.2. Monoclonal phage ELISA

In the early stages of optimizing the panning process, we found that false positive phage clones posed a big challenge. In some cases none of the positive clones from monoclonal phage ELISA bound to SRP–SR specifically (data not shown). After we incorporated the modifications listed above (see Section 2.2), the results improved significantly. Fig. 1C and D show the ELISA plates of Tomlinson I and Tomlinson J libraries, and the controls. We found that a majority of both the Tomlinson I and Tomlinson J clones tested were positive. Some wells in the control ELISA plates also showed identifiable color, and these samples were

Table 1
ELIA of scFvs against SRP–SR and subunits

		1	2	3	4	5	6	7	8	9	10	11
		IA02	IB11	JA01	JA03	JA05	JB05	JB09	JC03	JD02	JG01	No scFV
A	SRP-SR	10.381	10.618	7.79	9.442	10.229	11.66	10.496	11.658	11.634	10.258	0.212
B	FfH	0.469	10.952	12.761	0.181	0.524	70.602	0.145	0.352	0.203	0.233	0.226
C	hNG	0.146	10.7	9.779	0.158	0.398	10.273	0.171	0.176	0.328	0.263	0.194
D	hM	0.208	0.176	0.285	0.356	0.518	0.425	0.186	0.197	0.255	0.328	0.268
E	FtsY	0.224	0.243	10.142	9.6	10.618	8.876	1.3	14.119	12.686	14.677	0.17
F	yNg	0.228	0.24	19.658	12.687	11.668	13.657	1.299	15.477	11.443	9.514	0.153
G	RNA	0.238	0.176	0.469	0.294	0.208	0.365	0.351	0.279	0.344	0.237	0.301
H	No antigen	0.246	0.166	0.531	0.396	0.444	0.363	0.381	0.352	0.348	0.407	0.202

Values in each well is absorbance at 450 nm. Highlighted wells showed color visible to naked eyes.

immediately deleted from the list of positive clones. We also performed ELISA tests of clones from the original Tomlinson I and Tomlinson J libraries (no panning). While some wells in the Tomlinson J plate gave weak signals, neither the Tomlinson I plate nor either control plate showed any signal (data not shown). All of the monoclonal phage ELISA results confirmed that our improved panning protocol was successful in enriching phage clones that specifically bind to SRP–SR while simultaneously reducing false positive clones.

3.3. Sequence analysis

From 90 clones sequenced, a total of 10 different sequences were obtained. Two were from Tomlinson I: IA02, IB11. Eight were from Tomlinson J: JA01, JA03, JA05, JB05, JB09, JC03, JD02 and JG01. Similar to other reports utilizing Tomlinson phage libraries (Yan et al., 2004; Barderas et al., 2006; Marcus et al., 2006), many clones contain premature amber codons. In our case, nine out of 10 clones have amber codons (data not shown). These extra premature amber codons, while still allowing scFv-containing phage production in TG1 cells, prevent the expression of full-length scFvs following the provided protocol. Instead of changing the undesired amber codon (TAG) into a normal codon for glutamine (CAG), we chose a quicker strategy, as described above (see Section 2.5).

3.4. Expression and purification of scFvs

Soluble scFv was purified from the periplasmic fraction of RosettaBlue(DE3) cells transformed with each of the 10 phage clones. Fig. 2A shows the high purity of three purified scFvs; all other clones were purified to similar purity (data not shown). We found that the number of amber stop codons in the coding sequence affected the yield of scFvs. The clone that does not have an amber stop codon, IA02, could produce 0.5–1.0 mg scFv per liter of culture. The eight clones that have one amber stop codon gave similar yields, but the clone that contains two TAG codons, JA05, only produced 0.2–0.5 mg scFv per liter of culture.

3.5. ELISA with all scFvs

The absorbance reading of the ELISA plate is shown in Table 1. All of the scFv clones produced a strong signal with cross-linked SRP–SR, which further proved that they are true positive clones. Based on binding properties revealed by ELISA, the 10 scFvs could be classified into four groups. The first group has one member: IB11, which binds to hNG. The second group includes JA01 and JB05, which bind to both hNG and yNG, suggesting that they recognize the highly conserved region of hNG and yNG. The third group includes JA03, JA05, JC03, JD02 and JG01, which bind to yNG. The most interesting group is the last group: IA02 and JB09, which bind only to the intact complex, but not to any of its components. Although JB09 showed some interactions with yNG, the signal was weak compared to the signal observed with SRP–SR. This result suggested that group 4 recognizes structural features that are only present on the whole complex. Considering the diversity of the DNA and protein sequences of the scFvs, we speculate that different members in the same group may bind to different positions on the target molecules.
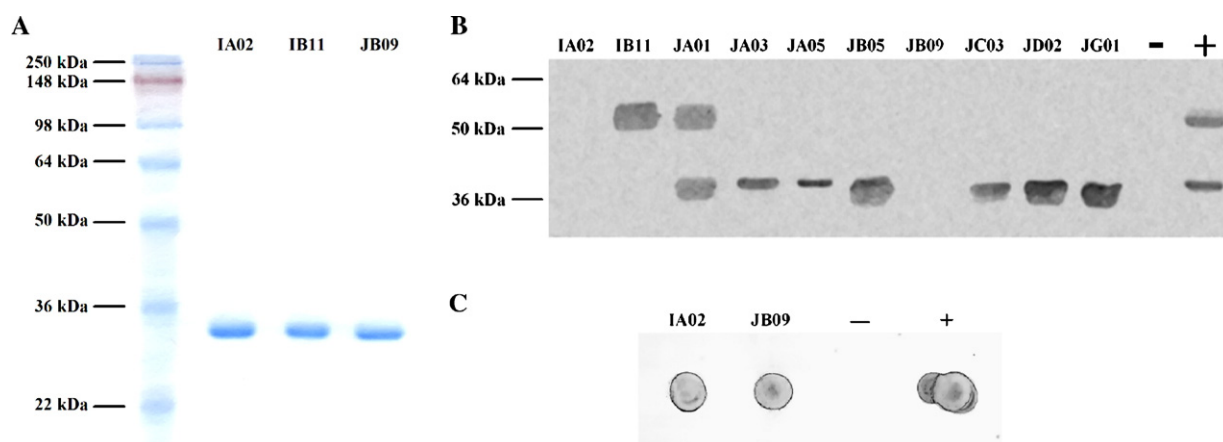

Fig. 2. Characterization of the scFvs. (A) SDS-PAGE analysis of purified scFvs. (B) Western blot with all scFvs. −: negative control where no primary antibody was used. +: positive control using mouse anti-SRP–SR serum as primary antibody (see Section 2.7). (C) Dot blot of IA02 and JB09. Controls are the same as (B).

3.6. Western blot with all scFvs

To test whether the scFv clones also bind to denatured Ffh and FtsY, we performed a Western blot. As shown in Fig. 2A, IA02 and JB09 did not react with either Ffh or FtsY, IB11 recognized Ffh only, JA01 bound to both Ffh and FtsY, JA03, JA05, JC03, JD02 and JG01 bound to FtsY only. The results of ELISA (Table 1) and Western blot (Fig. 2A) together suggested whereas IA02 and JB09 recognize structural features of SRP–SR, IB11, JA01, JA03, JA05, JC03, JD02 and JG01 recognize protein sequences within the complex. The only scFv that gave inconsistent results when assayed by ELISA and Western blot was JB05, which bound to both Ffh and FtsY in ELISA, but only with FtsY in Western blot.

3.7. Dot blot with IA02 and JB09

To further verify that IA02 and JB09 only bind to the intact, non-denatured complex, we also applied them in dot blot analysis (Fig. 2B). SRP–SR complex, when spotted on the membrane directly, should keep its ternary structure. Both IA02 and JB09 bound to the complex on the membrane, which confirmed that these two clones recognize conformational epitopes only present in the SRP–SR complex.

4. Conclusions

Although antibody phage display has been widely used, when it was employed to produce antibodies that target subunits of a macromolecular complex, the success rate was less than 1% (Rubinstein et al., 2003). By keeping the selection target, in our case the SRP–SR complex, the only constant substance in the panning process, and gradually increasing the stringency, we reduced the number of false positives and obtained a good pool of true positive clones. 10 distinct positive clones were identified from 176 clones screened in monoclonal phage ELISA. 80% of the positive clones recognize not only the intact complex, but also its subunits in different assays, while 20% of the positive clones only recognize the intact complex. Therefore, the epitope diversity of the positive clones is excellent.

Extra amber codons within the coding sequences of scFvs is a prevalent problem when using antibody phage display (Yan et al., 2004; Barderas et al., 2006; Marcus et al., 2006), which delays the production and characterization of scFv clones. We devised a new approach to circumvent this problem. Subcloning the scFv coding sequences to a new expression vector by restriction digestion and ligation is much easier and faster than mutagenesis approaches. Not only can our approach be used to the scFv clones one by one, but also be used to a pool of clones in a high throughput environment. We demonstrated that the pET26b–RosettaBlue(DE3) system expressed scFvs very well and all scFvs purified were soluble and fully functional.

Monoclonal antibodies are very useful in the study of the structure and function of large macromolecular complexes. We have devised a fast and efficient protocol to select and produce such antibodies with antibody phage display, which will be helpful to a wide range of research projects.

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