Cell-free immunology: construction and in vitro expression of a PCR-based library encoding a single-chain antibody repertoire

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Received 17 December 1998

Abstract A novel cloning-independent strategy has been developed to generate a combinatorial library of PCR fragments encoding a murine single-chain antibody repertoire and express it directly in a cell-free system. The new approach provides an effective alternative to the techniques involving in vivo procedures of preparation and handling large libraries of antibodies. The possible use of the described strategy in the ribosome display is discussed.

Key words: Single-chain antibody; Polymerase chain reaction library; Cell-free translation; Ribosome

1. Introduction

A notable advance has been made in recent years in constructing and harnessing large display libraries of antibodies. These libraries are used either for selecting new antibodies of certain antigen specificity or for improving binding affinities of antibody molecules. The key feature of all display techniques designed for selection of proteins is a physical linkage between a polypeptide chain ('phenotype' moiety) and its encoding message ('genotype' moiety). To realize this general concept, several experimental approaches have been applied to antibody selection beside the classical hybridoma technique. These approaches are mostly based on in vivo expression of antibody repertoires linked to viral [1–3] or cellular [4] surfaces. Displaying single-chain antibodies on prokaryotic [5] and eukaryotic [6] ribosomes has recently been demonstrated to be a promising cell-free alternative to the in vivo selection techniques. This novel methodology was realized due to the following experimental facts: (1) in vitro translation of mRNA lacking a stop codon results in a stable complex of the ribosome with both nascent polypeptide and its encoding message [7] and (2) the nascent polypeptide can attain its functionally active conformation in the ribosome-bound state [8–10]. The ribosome display is believed to have several potential advantages over other display techniques [11]. The ability of the ribosome display method to provide antibody selection has been shown, however, only with model systems containing some amount of a target single-chain antibody diluted with an overwhelming excess of competing antibody fragments. Further development of this technique for selecting antibodies from large repertoires requires a general strategy of library construction and its subsequent cell-free expression to be worked out. Here we demonstrate that a library of PCR fragments encoding a mouse single-chain antibody repertoire can be prepared without cloning procedures in a format suitable for its immediate expression in vitro. The strategy has potential to be further used in antibody display on the ribosome.

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; K, kappa light chain; NBT, 4-nitroblue tetrazolium chloride; PCR, polymerase chain reaction; scAb, single-chain antibody; SDS, sodium dodecyl sulfate; SOE, splicing by overlap extension; V_H, heavy chain variable domain

2. Materials and methods

2.1. Reverse transcription and PCR

Total RNA was isolated from homogenized spleens of two non-immunized mice by guanidinium thiocyanate-phenol-chloroform extraction [13]. The preparation was used for reverse transcription in a mixture containing 10 μg of the isolated RNA, 300 U of M-MLV reverse transcriptase (Promega) and 15 pmol of $p(dT)_{15}$ primer (Boehringer) in 30 μl of the reaction mixture. After 1 h incubation at 42°C, the mixture containing synthesized cDNA was divided into two 15 μl aliquots that were used for separate PCR amplifications of heavy chain variable fragments (V_H) and kappa light chain fragments (K). Both amplification reactions were carried out in 150 μl of standard PCR mixture containing 3 U of Taq DNA polymerase (Pharmacia). To amplify V_H fragments, the upstream oligonucleotide primer $V_h$up 5′-GAGGTCCAGCT(G/T)CTCGAGTC(A/T)GG-3′ and the downstream primer $V_h$down 5′-CAGAAGAAACGGTAACAGTGGTGCCTTGGCCCCA-3′ were added to a final concentration of 0.5 μM each. The mixture for K fragment amplification contained $V_K$up primer 5′-GAGCTCCAGATGACCCAGTCTCC-3′ and $C_K$down primer 5′-GTAGAATTCACACTCATTCCTGTTGA-3′ at 0.5 μM each. Both reactions were cycled 30 times (45 s at 94°C, 45 s at 45°C and 45 s at 72°C). The synthesized PCR fragments were purified using agarose gel electrophoresis and further used to construct the scAb library.

2.2. Plasmid construction

Phage vector M13RV1 (a gift of Dr. N.I. Matvienko, Institute of Protein Research, Pushchino) was derived from M13mp19 by replacement of the *Bam*HI-*Eco*RI polylinker fragment with the cassette 5′-GGATCCGAAGACCCGGGTCTTCGAATTC-3′ [12], containing two *Bbv*II sites (underlined) separated by a *Sma*I site. The M13RV1 vector cleaved with *Sma*I was used to clone a synthetic double-stranded linker fragment 5′-GGCACCACTGTTACCGTTTCTTCTGGTGGCGGCGGTTCTGGCGGTGGCGGTTCTGGTGGCGGTGGTTCTGAGCTCCAGATGACCCAGTCT-3′ encoding $(SerGly_4)_3$ peptide (underlined) flanked by sequences complementary to the 3′ part of V_H and the 5′ part of K fragments. The *Sal*I-*Eco*RI linker fragment of the resultant recombinant phage was then inserted into pTZ19R vector (Fermentas) at *Sal*I-*Eco*RI sites to give pTZlink plasmid. The plasmid was used as a source of the linker fragment for the PCR assembly reaction. The fragment was excised by treatment with *Bbv*II restriction endonuclease (prepared in the Institute of Protein Research, Pushchino), gel-purified and used for the library construction.

2.3. Generation of the library of scAb PCR fragments

PCR fragments encoding the single-chain antibody repertoire were prepared using splicing by overlap extension (SOE) technique [1,14].

PII: S0014-5793(99)00055-1

For this purpose, 300 ng of V_H and 500 ng of K fragments were mixed together with 200 ng of linker fragment in 50 μl of PCR mixture containing 1 U of Taq DNA polymerase. No oligonucleotide primers were added to the mixture. The reaction was cycled 8 times (1 min at 95°C, 1 min at 54°C and 1.5 min at 72°C) to splice the fragments together. The resulting scAb-encoding products were diluted five-fold into a fresh PCR mixture containing 0.25 μM of each T7V_H and $C_K$myc primers and amplified by 25 PCR cycles consisting of 1 min at 94°C, 1 min at 64°C and 1.5 min at 72°C. The upstream primer, T7V_H (52-mer), had the sequence 5′-taatacgactcactataGGG*AAGCTT*GCCACCATGGTCCAGCT(G/T)*CTCGAG*TC-3′, and included a T7 RNA polymerase promoter (shown in lower case) and a *Nco*I restriction site (underlined). The primer also comprised the ATG translation initiation codon in the optimum Kozak context [15], followed by 17 nucleotides overlapping the 5′ ends of V_H fragments. *Hin*dIII and *Xho*I sites of the primer are shown in italics.

The downstream primer, $C_K$myc (58-mer), had the sequence 5′-cagatcctcttctgagatgagtttttgttcTGCGGCCGCGTA*GAATTC*ACACTCATTC-3′, and contained the myc-tag sequence (lower case) [16], separated by a *Not*I site (underlined) from the 19 nucleotide region complementary to the 3′ ends of kappa chain fragments. The primer also included a *Eco*RI site (italicized).

2.4. Preparation of scAb mRNA

Messenger RNA was prepared by in vitro transcription [17] of the PCR-generated scAb genes with T7 RNA polymerase. The transcription reaction was carried out in 50 μl of 120 mM HEPES-KOH, pH 7.5 buffer, containing 24 mM $MgCl_2$, 1 mM spermidine, 20 mM DTT, 5 mM of each ribonucleoside 5′-triphosphates (Boehringer), 20 U of RNasin (Promega), 200 U of T7 RNA polymerase (prepared in the Institute of Protein Research, Pushchino) and 2 μg of the scAb-encoding PCR products. The mixture was incubated at 37°C for 2 h and then stopped with chloroform extraction. The transcript was purified by 3 M LiCl precipitation, re-precipitated with ethanol and dissolved in sterile water [18]. Homogeneity of the mRNA preparation was checked with electrophoresis in 5% PAG containing 7 M urea.

2.5. In vitro translation

Cell-free translation was performed in 70% nuclease-treated reticulocyte lysate (Promega). The reaction volume was 20 μl and the translation was carried out at 30°C for 1 h in the presence of 20 μM [^{14}C]leucine (specific activity 305 mCi/mmol, Amersham). Translation was stopped by addition of 5 μl of 0.25 M NaOH, 0.1 M EDTA, and the mixture was incubated for 15 min at 30°C to hydrolyze peptidyl-tRNA bonds. Aliquots from the alkaline-treated translation mixture were subjected to SDS-PAGE analysis on a 12.5% gel according to Laemmli [19]. Low range blue prestained SDS-PAGE standards (Bio-Rad) were used to estimate the molecular weights of the proteins synthesized. Radioactivity in the gel was detected by autoradiography with Hyperfilm-MP (Amersham). For the immunoblot analysis, proteins were electrotransferred from the SDS-PAGE gel to the PVDF membrane (Immobilon-P, Millipore). The membrane was blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 2% casein and incubated with alkaline phosphatase-conjugated goat antibody specific to mouse IgG Fab fragments (Sigma). After washing with TBST, the membrane was developed in TSM buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM $MgCl_2$) containing 0.033 mg/ml of NBT and 0.165 mg/ml of BCIP (both from Sigma) for 30 min at room temperature.

3. Results

An expressible library of PCR fragments encoding a single-chain antibody repertoire was constructed using the PCR assembly technique according to the scheme shown in Fig. 1. Spleen tissue of non-immunized mice was used as starting material. V_H and K fragments were first amplified in PCR with corresponding immunoglobulin-specific primers. The primers were designed according to the strategy described in [20,21]. A low annealing temperature (45°C) was used in both amplification reactions to ensure mismatch priming and, as a result, appropriate representation of mouse V_H and K repertoires. Gel-purified V_H and K fragments were PCR-assembled as previously described [1,14]. The resultant V_H/K genes encoding for scAb were further amplified using

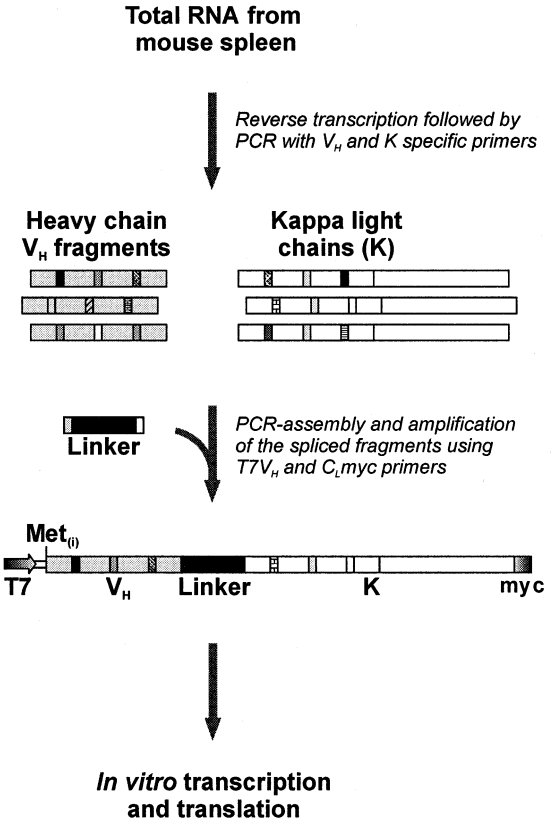

Fig. 1. General scheme of generation of PCR library encoding scAb.

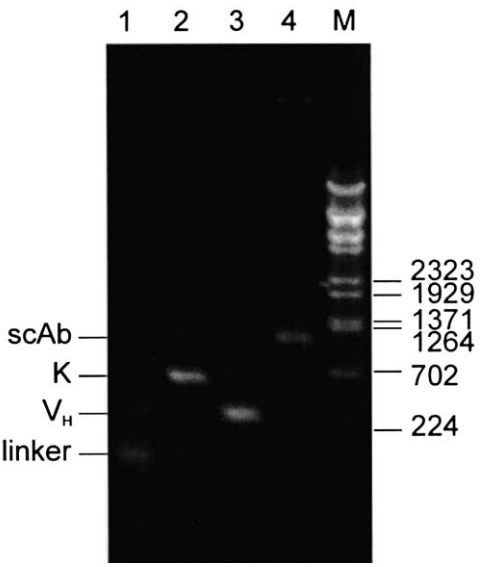

Fig. 2. PCR assembly of scAb genes from DNA fragments. Electrophoresis of DNA fragments was carried out on 1% agarose gel with subsequent ethidium bromide staining. Lanes: 1, small *Bbv*II fragment of pTZlink plasmid (linker fragment); 2, purified kappa light chain fragments; 3, purified heavy chain variable domain fragments; 4, assembled scAb genes; M, 0.5 μg of λDNA digested with *Bst*EII.

specially designed primers. The upstream primer ($T7V_H$) introduced the T7 RNA polymerase promoter and all necessary translation initiation signals and the downstream primer ($C_K$myc) introduced the myc-tag sequence [16] for detection of the relevant protein products. No stop codon was present in the $C_K$myc primer in the scAb open reading frame.

The result of the scAb assembly experiment is shown in Fig. 2. The length of the final product (approximately 1.1 kb) is in good agreement with the expected size of correctly spliced scAb fragments: the size of V_H fragments is 0.35 kb and that of K chains is 0.65 kb, whereas the total length of all the auxiliary sequences (T7 promoter, 5′ untranslated region, linker and myc-tag) is approximately equal to 0.1 kb. No specific fragment of 1.1 kb was produced in control assembly reactions lacking either V_H, K, or linker fragment (not shown).

The scAb-encoding PCR products were used without gel purification for in vitro mRNA synthesis with T7 RNA polymerase. Electrophoretic analysis of the transcript revealed a single band of the expected size (Fig. 3a). The synthesized mRNA was translated in nuclease-treated rabbit reticulocyte lysate system. After incubation at 30°C for 1 h, an aliquot from the translation mixture was subjected to SDS-PAGE (12.5% gel) followed by autoradiography (Fig. 3b). The products of the scAb mRNA translation migrated as a singe band with a molecular weight of approximately 40 kDa. The control experiment without mRNA did not contain any radioactive bands. The molecular weights of the newly synthesized

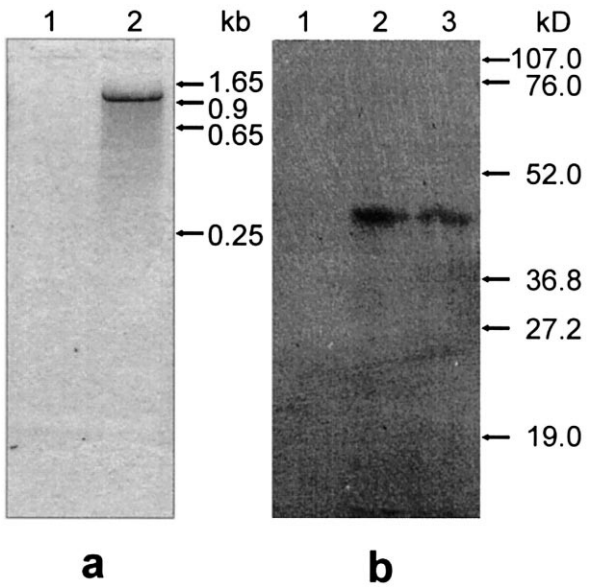

Fig. 3. In vitro transcription and translation. a: Electrophoretic analysis of in vitro transcription products was carried out in 5% PAGE containing 7 M urea. The gel was stained with toluidine blue to visualize RNA bands. Lanes: 1, control transcription without DNA template; 2, transcripts obtained from assembled scAb genes. Arrows show positions of molecular weight standards. b: SDS-PAGE of the cell-free translation products. 5 μl aliquots were removed from translation mixtures after 60 min incubation at 30°C and subjected to electrophoresis in 12.5% polyacrylamide gel followed by autoradiography. Lanes: 1, the control reaction without mRNA; 2, translation mixture programmed with 50 μg/ml of the scAb mRNA; 3, translation mixture containing 25 μg/ml of the scAb mRNA. Arrows indicate positions of molecular weight standards.

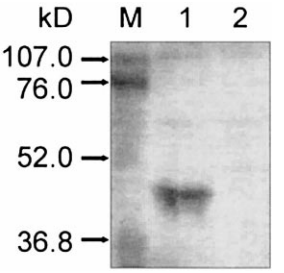

Fig. 4. Immunoblot analysis of the cell-free translation products. Cell-free translation products were fractionated by SDS electrophoresis as described in Fig. 3b, electrotransferred to PVDF membrane and stained with alkaline phosphatase-conjugated goat antibody specific to mouse IgG Fab fragments. Lanes: 1, scAb mRNA translation products; 2, the control reaction lacking mRNA. Positions of molecular weight standards are shown with arrows.

polypeptides are consistent with those theoretically calculated for the full-length V_H/K single-chain antibodies.

The following experiment was carried out in order to identify the newly synthesized polypeptides. It was assumed that V_H/K single-chain antibodies must share common antigenic determinants with antibody Fab fragments. Accordingly, identification of scAb polypeptides synthesized in vitro was done with commercially available Fab-specific antibodies conjugated with alkaline phosphatase. A band of approximately 40 kDa was detected by immunoblot analysis of the translation mixture programmed with scAb mRNA, whereas no specific signal was detected in the control mixture without mRNA (Fig. 4). This finding strongly suggests the cell-free synthesized polypeptides to be correctly engineered single-chain antibodies.

The cell-free synthesis of single-chain antibodies was also analyzed by TCA precipitation using [^{14}C]leucine as the labelled amino acid. Leucine residues were calculated to comprise approximately 7% of the total amino acid residue content of murine V_H and K polypeptide sequences from the 'Kabat Database of Sequences of Proteins of Immunological Interest' (http://immuno.bme.nwu.edu/ and [22]). Taking into account the estimated leucine content and TCA precipitation data, the total yield of synthesized antibodies was found to be 53 pmol/ml of the translation mixture under optimal conditions.

4. Discussion

The strategy reported here provides an effective cloning-independent way of constructing large combinatorial libraries encoding single-chain antibodies that can be directly expressed in a cell-free system. All the described procedures, from the purification of total RNA of an animal source to the cell-free expression of the scAb genes, take only 2 days. The prepared PCR library can be transcribed into V_H/K single-chain antibody-encoding messages lacking stop codons in the open reading frame. A model mixture of PCR fragments of a similar design has been shown previously to be suitable for displaying scAb on the eukaryotic ribosomes from rabbit reticulocyte lysate [6]. Consequently, the ribosome display procedure could be adopted with no or few modifications to the entire combinatorial library of scAb fragments. As follows from the estimated antibody yield, the procedure described here can, in principle, generate a library comprising

3.2×10^{13} molecules/ml of the translation mixture. The library described here exceeds in capacity the phage display libraries constructed from unimmunized animals [23,24]. These lower capacity libraries were demonstrated previously to yield single-chain antibodies of various antigen specificity upon selection. This gives a good clue for further utilization of the larger libraries constructed from healthy human donors or non-immunized animals by the PCR technique presented here. Once generated, the library can provide a vast variety of antibodies to be selected with the ribosome display procedure. After being selected from the library, the genes of single-chain antibodies of desired antigen specificity could be cloned into a plasmid vector for further sequencing and expression in vivo. In the library reported here, there are several restriction sites available for cloning on the 5′ end (*Hin*dIII, *Nco*I, and *Xho*I) and on the 3′ end (*Not*I and *Eco*RI) of each scAb gene. Viability assessment of the proposed scheme requires further experiments.

Acknowledgements: We thank N.I. Matvienko for donating the vector M13RV1 and restriction endonuclease *Bbv*II, A. Golubtsov and G. Johnson for the database computer analysis and L. Vasiljeva for gel photography. We are also grateful to R.M. Horton for his helpful advice on the SOE technique.

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