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Selection of antigenic markers on a GFP-Cκ fusion scaffold with high sensitivity by eukaryotic ribosome display

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Abstract

Ribosome display is a cell-free system permitting gene selection through the physical association of genetic material (mRNA) and its phenotypic (protein) product. While often used to select single-chain antibodies from large libraries by panning against immobilized antigens, we have adapted ribosome display for use in the 'reverse' format in order to select high affinity antigenic determinants against solid-phase antibody.

To create an antigenic scaffold, DNA encoding green fluorescent protein (GFP) was fused to a light chain constant domain (Cκ) with stop codon deleted, and with 5′ signals (T7 promoter, Kozak) enabling coupled transcription/translation in a eukaryotic cell-free system. Epitopes on either GFP (5′) or Cκ (3′) were selected by anti-GFP or anti-Cκ antibodies, respectively, coupled to magnetic beads. After selection, mRNA was amplified directly from protein–ribosome–mRNA (PRM) complexes by *in situ* PCR followed by internal amplification and reassembly PCR. As little as 10 fg of the 1 kb DNA construct, i.e. approximately 7500 molecules, could be recovered following a single round of interaction with solid-phase anti-GFP antibody. This platform is highly specific and sensitive for the antigen–antibody interaction and may permit selection and reshaping of high affinity antigenic variants of scaffold proteins.

Keywords: Ribosome display; Aptameric GFP; Aptamer; *In vitro* protein display; Protein–protein interaction

Ribosome display is a well-validated, cell-free system based on genotype/phenotype linked selection of proteins and their encoding mRNA, following binding to ligands immobilized on solid surfaces [1–3]. In ribosome display, deletion of the termination codon from the encoding DNA causes stalling of the ribosome such that the nascent translated protein remains attached to its mRNA as a ternary protein–ribosome–mRNA (PRM) complex. After affinity selection of the protein component of the PRM, the mRNA can be amplified by RT-PCR and the interacting gene cloned. In principle, this platform addresses protein–protein interactions of all categories, and hence could be used to reshape a substrate or enzyme inhibitor or modify epitopes for antibody recognition. While often used to select single-chain antibodies from large libraries by panning against immobilized antigens, we have adapted ribosome display for use in the 'reverse' format in order to select high affinity antigenic determinants against solid-phase antibody.

Abbreviations: PRM, protein–ribosome–mRNA complex; ARM, aptamer–ribosome–mRNA complex; Cκ, constant region of kappa light chain; rd, ribosome display; GFP, green fluorescent protein; FcεRIα, alpha receptor of high affinity IgE Fc receptor; nt, nucleotide.

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Passive monoclonal antibody (MAb)-directed therapy is used therapeutically to neutralize effector molecules such as cytokines and IgE and to alleviate certain inflammatory diseases [4]. There would be a distinct advantage in reshaping the antigens bound to a protective antibody, since knowledge of their structure can offer the alternative modality of active immunotherapy.

We determine herein with the reverse format the specificity and sensitivity of antigenic epitopes expressed by a GFP-Cκ ribosome display enabled construct for antibodies bound to the solid phase in ribosome display, using conserved major epitopes on the display scaffold.

Approximately, 10 fg DNA of this construct, or about 7500 molecules, was efficiently transcribed and translated and PRM complexes selected in the eukaryotic system. This provides the possibility that a library as large as 10^9 of closely related groups of antigenic variant constructs (10 μg input DNA) may be selected for high affinity ligands.

Materials and methods

Reagents. Oligonucleotide synthesis was by Allele Biotechnology (San Diego, CA). Plasmid $pGFP_{UV}$ was from Clontech (Mountain View, CA). RNase-free DNase I, T4 DNA ligase, and Vent DNA polymerase were from NEB. PCR, Omniscript RT and Qiax-II kits were from Qiagen (Valencia, CA). His-Select Nickel affinity gel was from Sigma–Aldrich (St. Louis, Mo). TNT quick coupled transcription/translation systems and transcend charged biotinylated lysine were from Promega (Madison, WI). Hydrophobic tosyl-activated Dynabeads were from Dynal (Oslo, Norway). Nunc Maxisorp™ 96-well plates were from Fisher Scientific (Pittsburgh, PA). Rabbit anti-mouse κ antibody was from Rockland (Gilbertsville, PA 19525) and Sigma–Aldrich (St. Louis, MO). MAb anti-GFP antibody (B34) was from Convas (Richmond, CA).

Cell-free expression and labeling. For monitoring the *de novo* translated product, 1 μl of biotinylated charged lysine Transcend tRNA was added to the TNT system. For a typical 50 μl of reaction, 40 μl TNT Quick Master Mix was used, followed by 2.5–5 μl of PCR generated DNA templates, corresponding to 100 ng to 1 μg purified DNA, and 1 μl of charged lysine, and *de novo* translated product detected by strepavidin-HRP and substrate. The specificity of the translated protein was ascertained also by immunoblot with anti-Cκ antibody.

Ribosome display construct and method for selection. The gene-C construct was designed for eukaryotic ribosome display as described [2]. Primers used for PCR and RT-PCR are listed as sequences in Table 1 and their annealing positions shown in Figs. 1–4. Ribosome display constructs were generated by fusion of the gene of interest to the mouse Cκ domain as spacer [2].

The generation of DNA encoding GFP was carried out by PCR amplification using primers Ym1 and gfp-r-not1 on the plasmid $PGFP_{UV}$ as template (Fig. 1A). DNA fragments encoding Cκ from total RNA extracted from hybridoma cells from rodent anti-DNP IgE-producing hybridoma 16.82 (κ, ε) (Fig. 1B). After RT on the total RNA using random primers, the first-stranded cDNA was amplified by PCR using primers ck-f not1 and ck-r pst for Cκ (Fig. 1C). To generate GFP-Cκ fusions for ribosome display, the PCR fragments encoding GFP were assembled with Cκ, followed by amplification of the products using primers Ym1 and cκ-r pst (Fig. 1D).

Ribosome display was carried out as follows: PCR construct DNA was added to 50 μl rabbit reticulocyte lysate at 30 °C for 60 min. On completion of the reaction, the mixture was kept at 4 °C or on ice if selection was pursued immediately. Hydrophobic tosyl-activated Dynabeads (2.8 mm with *p*-toluene sulfonyl, 1.3 g/ml) were coupled with anti-GFP antibody or anti-Cκ antibody, according to the manufacturer's instructions, overnight at 4 °C. For selection on beads coupled with anti-GFP antibody or anti-Cκ antibody, the translation mixture was diluted on ice at 1:8 ratio with 1× phosphate-buffered saline (PBS) containing 5 mM Mg acetate before adding to 16–24 μl beads and incubated at 4 °C for 2 h.

After washes, DNA was recovered by one of the following methods: (i) RT-PCR was performed directly on the ribosome complexes (*in situ* recovery) with a 3′ primer annealing upstream of the stalled ribosome [5]; or (ii) mRNA was eluted from the ribosome complexes with 10 μl PBS/20 mM EDTA, followed by purification prior to RT-PCR [3]. To eliminate possible input DNA contamination, 10 U RNase-free DNase I (NEB) was added to eluted mRNA or complexes and incubated for 20 min at 37 °C (i), and ck-r pst for method (ii). Follow-up full length assembly PCR was performed with 5′ primers, Ym1 or Ym-T7 and 3′ primer cκ-r pst for 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min.

Alternatively, to enhance the recovery of DNA, a partial RT-PCR fragment after ribosome display, internal primers, rd4-504f, and rd4-922r were synthesized as a fragment encompassing the critical region of loop 6 and 8/9 of GFP intended for an inserted peptide library. 5′ stuffer fragment was prepared with Ym1 or Ym-T7 with rd4-574r. The RT-PCR products were then assembled into the full-length ribosome display enabled cassette by PCR overlapping the added Cκ domain with terminal primers Ym-T7 and cκ-r pst.

Results

Transcription/translation yield of GFP-Cκ fusion construct

To compare the efficacies of the sequences enabling transcription and translation, the 5′ regulatory regions

Table 1
Nomenclature sequence

Ym-T7	gaaactctagaTAATACGACTCACTATAgagggagccaccatgggtaaaggagaagaac
T7 ext	agatctcgatcccgcgaaatTAATACGACTCACTATA
Ym1	T7 ext + gagggagccaccatgggtaaaggagaagaac
Ym2	T7 ext + gggagaccacaacggtttcccgccaccatgggtaaaggagaagaac
Ym1.25	T7 ext + gagggacaaaccatgagtaaaggagaagaacttttcactg
P.ext	T7 ext + gggagaccacaacggtttcccgaatacaagcttgcttgttctttttgcag
	aagctcagaataaacgctcaactttggcagatctaccatgggtaaaggagaagaac
S.ext	T7 ext + gggacaattactatttacaattacaatggggtaaaggagaagaac
gfp-r not1	gaagcggccgcctttgtagagctcatc
cκ-f not1	aatggcggccgctgatgctgcacca
cκ-r pst	aattgctgcagaccacactcattcctgttgaagct
rd4-504-f	aagatggaaacattctcggac
rd4-922-r	aagttgttcaagaagcacacg
rd4-575-r	gtctgccgtgatgtatac