Antibody–ribosome–mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites

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Received June 9, 1997; Revised and Accepted October 4, 1997

ABSTRACT

We describe a rapid, eukaryotic, in vitro method for selection and evolution of antibody combining sites using antibody–ribosome–mRNA (ARM) complexes as selection particles. ARMs carrying single-chain (VH/K) binding fragments specific for progesterone were selected using antigen-coupled magnetic beads; selection simultaneously captured the genetic information as mRNA, making it possible to generate and amplify cDNA by single-step RT-PCR on the ribosome-bound mRNA for further manipulation. Using mutant libraries, antigen-binding ARMs were enriched by a factor of 10^4–10^5-fold in a single cycle, with further enrichment in repeated cycles. While demonstrated here for antibodies, the method has the potential to be applied equally for selection of receptors or peptides from libraries.

There is considerable interest in the display of large libraries of proteins and peptides and means of searching them by affinity selection. The key to genetic exploitation of a selection method is a physical link between individual molecules of the library and the genetic information encoding them. A number of cell-based display methods are available, e.g. on the surfaces of phages (1,2), bacteria (3) and animal viruses (4). Display of a peptide library on prokaryotic polysomes in a cell-free system has also been described (5) and recently this method was modified to display and select single-chain antibody fragments (6).

Here we report a eukaryotic, in vitro method for rapid selection and evolution of antibody combining sites using antibody–ribosome–mRNA (ARM) complexes as selection particles (Fig. 1). The concept is based on two experimental results: (i) single-chain antibodies are functionally produced in vitro in rabbit reticulocyte lysates (7) and (ii) in the absence of a stop codon, individual nascent proteins remain associated with their corresponding mRNA as stable ternary polypeptide–ribosome–mRNA complexes in cell-free systems (8,9). We have applied these findings to a strategy for preparing libraries of ARM complexes and have selected ARMs carrying a specific combining site using antigen-coupled magnetic beads. Since selection simultaneously captures the relevant genetic information (mRNA), cDNA can be generated and amplified by single-step RT-PCR on the ribosome-bound mRNA for sequencing, expression and further manipulation.

From the anti-progesterone antibody DB3, we have previously constructed a single-chain fragment (VH/K) comprising the heavy chain variable domain (VH) linked to the complete κ light chain (K) (10,11). Using the ‘megaprimer’ PCR method (12), DB3 VH/K mutants were produced at VH positions H100 or H35 (He,M. and Taussig,M.J. unpublished), binding site contact residues for progesterone (13). DB3R is a mutant in which tryptophan H100 was substituted by arginine; when expressed from Escherichia coli, DB3R VH/K binds strongly to progesterone (K_a ∼10^9 M⁻¹) but has no affinity for testosterone or BSA. In contrast, mutants at H35 (DB3H35 VH/K) do not bind progesterone. Here we have employed DB3R and DB3H35 mutants to test the principle of ARM selection.

To generate VH/K DNA fragments for production of ARMs, PCR was performed using appropriate templates together with (i) an upstream T7 primer, containing the T7 promoter, protein initiation sequence and degenerate sequence complementary to mouse antibody 5′ sequences, and (ii) a downstream primer, D1, lacking a stop codon. The T7 primer sequence was

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For ARM selection, magnetic beads (Dynal) were coupled to bovine serum albumin (BSA), progesterone-11α-BSA, testosterone-3-BSA (Sigma) or purified rat anti-mouse κ antibody. Antigen- or anti-κ-conjugated beads (3 μl) were added to the translation mixture and transferred to 4°C for a further 60 min, with gentle vibration to prevent settling. Beads were recovered by magnetic particle concentrator (Dynal), washed three times with 50 μl cold, sterilised phosphate buffered saline (PBS), pH 7.4, containing 0.1% BSA and once with PBS alone. The beads were treated at 37°C for 15 min with DNase I (Promega) in 50 μl transcription buffer (Promega) containing 4 U of enzyme, followed by three washes with 50 μl PBS and resuspension in 10 μl nuclease-free water.

To produce and amplify cDNA from the mRNA of antigen-selected ARMs, RT-PCR was performed by adding 2 μl bead suspension to 23 μl RT-PCR mixture (either Access RT-PCR One Tube RT-PCR System, Promega or Titan  System, Promega or Titan ). PCR constructs from (1 ng to 1 μg) either purified by QIAquick (QIAGEN) or unpurified, were added to 20 μl of the TNT T7 Quick Coupled Transcription/Translation System (Promega) containing rabbit reticulocyte lysate with 0.02 mM methionine, and the mixture (25 μl) incubated at 30°C for 60 min.

For ARM selection, magnetic beads (Dynal) were coupled to bovine serum albumin (BSA), progesterone-11α-BSA, testosterone-3-BSA (Sigma) or purified rat anti-mouse κ antibody. Antigen- or anti-κ-conjugated beads (3 μl) were added to the translation mixture and transferred to 4°C for a further 60 min, with gentle vibration to prevent settling. Beads were recovered by magnetic particle concentrator (Dynal), washed three times with 50 μl cold, sterilised phosphate buffered saline (PBS), pH 7.4, containing 0.1% BSA and once with PBS alone. The beads were treated at 37°C for 15 min with DNase I (Promega) in 50 μl transcription buffer (Promega) containing 4 U of enzyme, followed by three washes with 50 μl PBS and resuspension in 10 μl nuclease-free water.

To produce and amplify cDNA from the mRNA of antigen-selected ARMs, RT-PCR was performed by adding 2 μl bead suspension to 23 μl RT-PCR mixture (either Access RT-PCR One Tube RT-PCR System, Promega or Titan  System, Boehringer Mannheim) containing the T7 primer (above) and a new downstream primer, D2, 5′-cggagaggtgctgctcatg-3′, designed to hybridise at least 60 nt upstream of the 3′-end of ribosome-bound mRNA. The use of D2 avoids isolating the mRNA from ARM complexes (Fig. 1). Products were analysed by agarose gel electrophoresis and sequenced.

For further cycles of ARM generation, PCR products were added directly to the TNT Transcription/Translation System. In a second cycle, the RT-PCR downstream primer, D3, 5′-ggggtagaagttgttcaag-3′, was designed to hybridise upstream of D2; similarly in the third cycle the primer D4, 5′-cttgagaggtgctgctcatg-3′, hybridising upstream of D3, was used. While the recovered DNA becomes progressively shorter in each cycle, full length Vβ/K can be regenerated in any cycle by recombinational PCR. The shortening only affects the constant domain of the light chain.

To demonstrate antigen-specific ARM selection, DB3R Vβ/K was translated in vitro and the ARM complexes exposed to magnetic beads coupled to BSA, progesterone-11α-BSA or testosterone-3-BSA. After RT-PCR, a single DNA fragment was detected only from progesterone-11α-BSA coupled beads (Fig. 2A, lanes 2, 4 and 6), consistent with the specificity of DB3R Vβ/K. Binding of the DB3R ARM was also specifically inhibited by free progesterone-11α-hemisuccinate, but not by related steroids which do not bind the intact antibody, confirming the specificity of the nascent antibody (data not shown). The recovered fragment was further confirmed as DB3R by sequencing. No bands were obtained when PCR alone was carried out on antigen-coupled beads after ARM selection (Fig. 2A, lanes 3, 5 and 7), or when the procedure was performed with non-translated DB3R mRNA (Fig. 2, lane 1). Thus, the band recovered by RT-PCR was due to amplification of mRNA selected via the combining site of DB3R and not from DNA contamination or mRNA carryover.

To investigate selection of a specific Vβ/K fragment from libraries, DB3R was mixed with the random DB3HE3 non-progesterone-binding mutants. When the DB3HE3 mutant library alone was displayed as ARMs, no DNA band was recoverable after selection with progesterone-11α-BSA beads (Fig. 2B, lane 5); translation of DB3HE3 was demonstrated by selection with beads coated with rat anti-κ antibody (Fig. 2B, lane 4). This was carried out as a side-by-side comparison with DB3R (Fig. 2B, lanes 2 and 3). When DNA mixtures containing DB3R and DB3HE3 mutants in ratios from 1:10 to 1:10^5 were displayed as ARM libraries, a band of Vβ/K size was in all cases recovered after a single cycle (Fig. 2C, lanes 1–5). Direct sequencing of PCR products before and after the ARM cycle confirmed the selection of DB3R. Thus, before selection DB3R was not detectable in the 1:10^3–1:10^5 ratio libraries, whereas after selection DB3R was the dominant species recovered from the 1:10^5 ratio libraries and comprised ~50% of the PCR product recovered from the 1:10^5 library. This data indicated that 10^4–10^5 fold-enrichment of this fragment was achieved in one ARM cycle.

While a 1:10^6 DB3R:DB3HE3 library did not produce a detectable RT-PCR band after one cycle (Fig. 3, lane 2), two further cycles of ARM generation and selection led to recovery of a Vβ/K band, with increasing intensity at each repetition (Fig. 3, lanes 3 and 4). Sequencing again confirmed the selection of the DB3R fragment.

Here we have demonstrated that the genetic information for an antibody fragment can be retrieved from ARM complexes, comprising nascent protein, ribosome and mRNA, after selection on immobilised antigen. Using ARM libraries, 10^5–10^6 fold enrichment of a specific combining site was obtained in a single cycle, with further enrichment in subsequent cycles. This is significantly greater than that reported (∼200-fold per cycle) for prokaryotic polysome display of antibody fragments (6). Since the TNT system contains ∼10^4 ribosomes/ml (supplier’s information), ARM libraries at least 10^12 in size should be achievable with the current protocol. Thus, ARM display may permit efficient isolation of antibodies from large libraries made from lymphocytes or...
Figure 3. Enrichment of DB3 R from a 1:10⁶ (DB3 R:DB3 H35) library by repeated ARM display cycles. Selection was with progesterone-11α-BSA coupled beads. Lane 1, 1 kb DNA marker; lane 2, RT-PCR after first cycle; lane 3, RT-PCR after second cycle; lane 4, RT-PCR after third cycle. The shortening of the band between cycles 2 and 3 is due to the use of different primers (D3 and D4, respectively).

generated in vitro. Mutations can be continuously introduced in repeated cycles without the need for DNA cloning steps; mutants with desired properties such as improved affinity can be sequentially selected, leading to evolution of desired combining sites.

This eukaryotic ARM display method is simple and rapid, each cycle being completed in 8 h, including analysis. A major advantage over polysome display (5,6) is that RT-PCR can be performed while mRNA is attached to the complex, avoiding time-consuming mRNA elution and purification and related losses. Although in this report we have only tested selection of antibody combining sites, the method could equally be applied to libraries of receptors or peptides, with potential applications in drug discovery (14).

ACKNOWLEDGEMENTS

We thank Dr Hong Liu for technical assistance, Dr G. Butcher for rat anti-mouse κ antibody and Mr J. Coadwell for artwork. M.Y.H. thanks the Babraham Institute for support.

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