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

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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 (V_H/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.

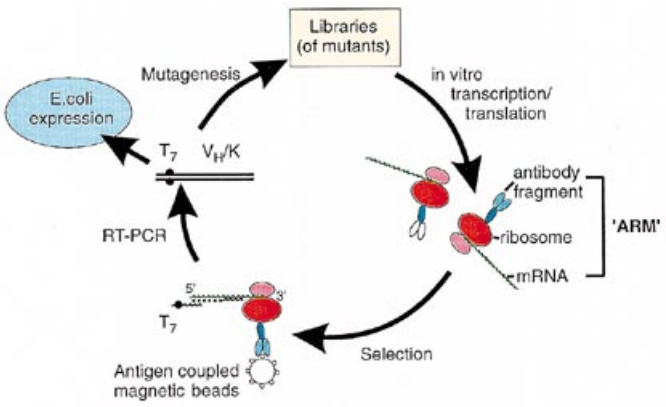

Figure 1. The ARM display cycle, showing the generation of an ARM library by mutagenesis of a V_H/K template, antigen-selection of a specific binding ARM and recovery of the genetic information by RT-PCR.

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the 'megaprimer' PCR method (12), DB3 V_H/K mutants were produced at V_H positions H100 or H35 (He,M. and Taussig,M.J. unpublished), binding site contact residues for progesterone (13).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

To generate V_H/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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5′-gcgcgaatacgactcactatagagggacaaaccatgsaggtcmarctcgagsagtcw-gg-3′ (s = c/g, m = a/c, r = a/g and w = a/t); the D1 primer was 5′-tgcactggatccaccacactcattcctgttgaagct-3′. V_H/K 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 System, Promega or Titan™ One Tube RT-PCR System, Boehringer Mannheim) containing the T7 primer (above) and a new downstream primer, D2, 5′-cgtgagggtgctgctcatg-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-aag-3′, was designed to hybridise upstream of D2; similarly in the third cycle the primer D4, 5′-ctggatggtgggaagatgg-3′, hybridising upstream of D3, was used. While the recovered DNA becomes progressively shorter in each cycle, full length V_H/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, $DB3^R$ V_H/K was translated *in vitro* and the ARMs 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 $DB3^R$ V_H/K. Binding of the $DB3^R$ ARM was also sensitively 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 $DB3^R$ 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 $DB3^R$ mRNA (Fig. 2, lane 1). Thus, the band recovered by RT-PCR was due to amplification of mRNA selected via the combining site of $DB3^R$ and not from DNA contamination or mRNA carryover.

To investigate selection of a specific V_H/K fragment from libraries, $DB3^R$ was mixed with the random $DB3^{H35}$ non-progesterone-binding mutants. When the $DB3^{H35}$ mutant library alone was displayed as ARMs, no DNA band was recoverable

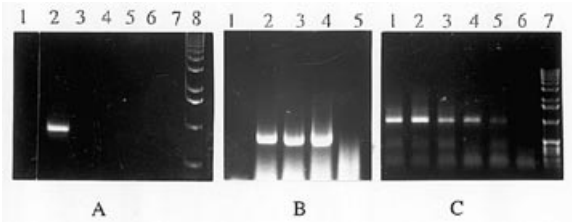

Figure 2. (**A**) Specific selection of $DB3^R$ ARMs by progesterone-11α-BSA-coupled beads. Lane 1, RT-PCR of non-translated $DB3^R$ mRNA selected by progesterone-11α-BSA beads; lane 2, RT-PCR of $DB3^R$ ARMs selected by progesterone-11α-BSA beads; lane 3, PCR of $DB3^R$ ARMs selected by progesterone-11α-BSA beads; lane 4, RT-PCR of $DB3^R$ ARMs selected by testosterone-3-BSA beads; lane 5, PCR of $DB3^R$ ARMs selected by testosterone-3-BSA beads; lane 6, RT-PCR of $DB3^R$ ARMs selected by BSA beads; lane 7, PCR of $DB3^R$ ARMs selected by BSA beads; lane 8, 1 kb DNA marker. (**B**) Non-binding of a $DB3^{H35}$ ARM library to progesterone-11α-BSA-coupled beads. Lane 1, RT-PCR of solution control; lane 2, RT-PCR of $DB3^R$ ARMs selected by rat anti-κ-coupled beads; lane 3, RT-PCR of $DB3^R$ ARMs selected by progesterone-11α-BSA beads; lane 4, RT-PCR of $DB3^{H35}$ ARMs selected by anti-κ beads; lane 5, RT-PCR of $DB3^{H35}$ ARMs selected by progesterone-11α-BSA beads. (**C**) Selection of $DB3^R$ from ARM libraries containing different ratios of $DB3^R$ and $DB3^{H35}$ mutants. Selection was with progesterone-11α-BSA coupled beads. Lane 1, ratio of $DB3^R$:$DB3^{H35}$ of 1:10; lane 2, $1:10^2$; lane 3, $1:10^3$; lane 4, $1:10^4$; lane 5, $1:10^5$; lane 6, $DB3^{H35}$ mutant library alone; lane 7, 1 kb DNA marker. The program for single-step RT-PCR was one cycle at 48°C for 45 min, followed by one at 94°C for 2 min, then 40 cycles consisting of 94°C for 30 s, 54°C for 1 min and 68°C for 2 min; finally one cycle at 68°C for 7 min was followed by 4°C.

after selection with progesterone-11α-BSA beads (Fig. 2B, lane 5); translation of $DB3^{H35}$ 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 $DB3^R$ (Fig. 2B, lanes 2 and 3). When DNA mixtures containing $DB3^R$ and $DB3^{H35}$ mutants in ratios from 1:10 to $1:10^5$ were displayed as ARM libraries, a band of V_H/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 $DB3^R$. Thus, before selection $DB3^R$ was not detectable in the $1:10^2$–$1:10^5$ ratio libraries, whereas after selection $DB3^R$ was the dominant species recovered from the $1:10^3$ and $1:10^4$ 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 $1:10^6$ $DB3^R$:$DB3^{H35}$ 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 V_H/K band, with increasing intensity at each repetition (Fig. 3, lanes 3 and 4). Sequencing again confirmed the selection of the $DB3^R$ 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, 10^4–10^5-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^{14} 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

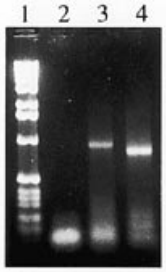

Figure 3. Enrichment of $DB3^{R}$ from a $1:10^{6}$ ($DB3^{R}$:$DB3^{H35}$) library by repeated ARM display cycles. Selection was with progesterone-11α-BSA coupled beads. Lane 1, 1 kb DNA marker; lane 2, RT-PCR after first cycle; lane 3, RT-PCR after second cycle; lane 4, RT-PCR after third cycle. The 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 continously 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).

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