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In vitro improvement of a shark IgNAR antibody by Qβ replicase mutation and ribosome display mimics *in vivo* affinity maturation

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Abstract

We have employed a novel mutagenesis system, which utilizes an error-prone RNA dependent RNA polymerase from Qβ bacteriophage, to create a diverse library of single domain antibody fragments based on the shark IgNAR antibody isotype. Coupling of these randomly mutated mRNA templates directly to the translating ribosome allowed *in vitro* selection of affinity matured variants showing enhanced binding to target, the apical membrane antigen 1 (AMA1) from *Plasmodium falciparum*. One mutation mapping to the IgNAR CDR1 loop was not readily additive to other changes, a result explained by structural analysis of aromatic interactions linking the CDR1, CDR3, and Ig framework regions. This combination appeared also to be counter-selected in experiments, suggesting that *in vitro* affinity maturation is additionally capable of discriminating against incorrectly produced protein variants. Interestingly, a further mutation was directed to a position in the IgNAR heavy loop 4 which is also specifically targeted during the *in vivo* shark response to antigen, providing a correlation between natural processes and laboratory-based affinity maturation systems.

Keywords: IgNAR; Affinity maturation; Shark antibody; Ribosome display; *In vitro* evolution; Immune evolution

1. Introduction

The vertebrate immune system produces high-affinity antibodies by the process of affinity maturation. In response to repeated contact with antigen, weakly binding antibodies are matured by hypermutation and cell proliferation, with mutations that introduce an increase in antigen affinity conferring a selective advantage on the lymphocyte. This highly dynamic cellular process of affinity discrimination occurs at the "B-cell synapse" and leads to clonal proliferation and differentiation of high-affinity antibody-bearing lymphocytes [1].

The *in vivo* process of affinity maturation can be mimicked *in vitro* by technologies which combine laboratory-based systems such as bacteriophage display with mutagenesis of antibody variable genes, followed by iterative rounds of selection on antigen [2]. Mimicking a highly evolved immune surveillance system is necessarily complex, and differing combinations of display systems (bacteriophage, cell surface, yeast, ribosome, *in vivo* compartmentalization) and mutation strategies (mutator cells, error-prone PCR, DNA shuffling) have been tested with varying degrees of success [3,4]. One recent mutagenesis system utilizes an error-prone RNA dependent RNA polymerase from Qβ bacteriophage to generate diversity, and allows coupling of randomly mutated mRNA templates directly to the translating ribosome ([5]; G.K., submitted for publication). The resulting complex, displaying the variant antibody domain, can then be selected against antigen and recovered intact, allowing subsequent amplification of the preferentially selected RNA molecule. Advantages of this tightly controllable, completely *in vitro* RNA mutagenesis approach include the ability to rapidly select for increased affinity and specificity through random sampling of a wider diversity of variants than generated by other mutagenesis methods.

Affinity maturation in the context of the Ig superfamily adaptive immune system finds it earliest expression in the

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Elasmobranchs (sharks, rays, skates). Reflecting this extended evolutionary history, sharks exhibit several unusual immune features, including an antibody repertoire which includes "classical" isotypes and the unique Immunoglobulin New Antigen Receptors (IgNARs) isoform [6,7]. Intact IgNARs are homodimers, consisting of two protein chains each with five or three constant domains ($C_{NAR}S$) and a single variable domain (V_{NAR}) [8]. Because the single V_{NAR} domains are effectively halved in their antigen-binding surface compared to for example IgGs, they compensate by utilizing long CDR3 loops which are significantly increased in length compared to those found in V_H-V_L antibodies; these are often stabilized by disulphide linkages either between the CDR1 and CDR3 loops or between framework and CDR3 regions [8,9]. In this sense, they are similar to the V_HH single domain antibodies found in camelids, though this isotype clearly retro-evolved from a conventional IgG-like antibody relatively recently in evolutionarily terms [10].

Because of their relatively simple structure and ease of manipulation as recombinant proteins in the laboratory, IgNAR variable domains represent ideal models systems for testing of *in vitro* affinity maturation techniques. For example, previously we reported the initial isolation of a V_{NAR} targeting the apical membrane antigen-1 (AMA1) of malarial (*Plasmodium falciparum*) parasites and its subsequent affinity maturation by error-prone PCR and bacteriophage selection techniques [11]. Two independent affinity-enhanced variants were selected, each exhibiting approximately 10-fold improvements in affinity, attributable to single residue changes within the extended CDR3 loop. In this study, we attempt to further affinity mature this V_{NAR} domain using the Qβ mutagenesis and ribosome display system described above. We show that the resulting mutations target exposed regions in the immune structure, and mimic affinity-matured variants seen in the natural *in vivo* immune response.

2. Experimental procedures

2.1. Equipment and reagents

Vent and Taq DNA polymerases and all restriction enzymes were purchased from New England Biolabs (Beverley, MA) and used according to the manufacturer's instructions. T4 DNA ligase was from Biotech (Australia). DNA fragment recovery and purification was by QIAquick Gel Extraction Kit, Qiagen (Germany). Small-scale preparations of DNA from *Escherichia coli* were by QIAprep Spin Miniprep Kit, Qiagen (Germany). DNA clones were sequenced on both strands using a BigDye terminator cycle sequencing kit (Applied Biosystems, USA) and a Perkin-Elmer Sequenator. Purified anti-FLAG antibody was immobilized onto Mini-Leak™ Low resin from Kem-En-Tec (Denmark) following the manufacturer's instructions, to generate anti-FLAG affinity resin.

2.2. Generating mutated RNA libraries with Qβ replicase

A 12Y-2 variant library was created by mutating the gene sequence at the RNA level using Qβ replicase (G.K., submitted for publication). Briefly, mRNA template suitable for Qβ replicase was transcribed from DNA template with T7 polymerase, DNase-treated to remove the DNA template (RQ1 DNase, Promega) and purified (RNeasy, Qiagen). One hundred nanograms of the mRNA template was pre-heated for 2 min at 95 °C in a thermocycler and permitted to slowly cool to room temperature. The RNA was mixed with 40 mM Tris–HCl (pH 7.9), 21 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 1 mM each of rCTP, rUTP, rGTP, and rATP, 2 U RNase inhibitor (Promega) and 200 nM Qβ replicase (prepared in house following the method of Moody et al. [12]) and incubated for a minimum of 120 min at 37 °C. The Qβ replicase amplified RNA was purified (RNeasy, Qiagen) to remove excess $MgCl_2$ prior to ribosome display.

2.3. Ribosome display

Ribosome display was performed essentially as outlined [13,14]. Two micrograms of heat denatured Qβ replicase mutated RNA was directly added to a 50 μL rabbit reticulocyte based translation reaction (Promega) following the manufacturer's recommendations. Translation was allowed to proceed for 25 min at 30 °C before diluting the translation mix with the addition of biotin-free skim milk (4%, w/v), PBS, and 5 mM $MgCl_2$. One nanomolar biotinylated AMA1 was added directly to the diluted translation mix which was subsequently rocked at 4 °C for 5 h prior to the addition of 2000 nM AMA1. The mix was rocked at 4 °C for a further 2 h (which constituted the competition phase). Ribosome complexes that remained attached to the biotinylated AMA1 were recovered with streptavidin coated magnetic beads (Dynal). Beads were subsequently washed three times with PBS containing 5 mM $MgCl_2$ and 0.1% Tween 20 and two times with PBS containing 5 mM $MgCl_2$ before being re-suspended in 40 μL of pre-heated dH_2O (55 °C) to disrupt the ribosome complexes. The beads were removed and the supernatant was used directly in RT-PCR to recover RNA using gene specific primers, the RT-PCR product cloned into expression vector pGC, and 500 individual clones analyzed for binding [15]. A second round of mutagenesis and ribosome display was performed on the best variant/s identified in round one, but incorporated increased selection pressure by increasing the competition phase to 16 h prior to RNA recovery. Following the second round of mutagenesis and panning, 500 individual clones were analyzed.

2.4. Expression and analysis of recombinant V_{NAR} proteins

Recombinant proteins were expressed into the bacterial periplasm and purified by affinity chromatography using an anti-FLAG antibody-Sepharose column [11]. Affinity purified protein was subjected to gel-filtration chromatography on a Superdex 200 column (GE Healthcare) equilibrated in HBS buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) for peak purification of V_{NAR} monomers. ELISA analysis was by standard techniques [9,11].

For analysis of V_{NAR} protein binding to AMA1, a BIAcore™ 1000 biosensor (BIAcore AB, Uppsala, Sweden) was used. AMA1 was immobilized onto a CM5 sensorchip at 25 μg/mL