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Research paper

A single-step procedure of recombinant library construction for the selection of efficiently produced llama VH binders directed against cancer markers

Damjana Kastelic [a,b], Snježana Frković-Grazio [c], Daniel Baty [d,1], Gilles Truan [a], Radovan Komel [b], Denis Pompon [a,*]

[a] CNRS, Centre de Génétique Moléculaire (CGM) FRE3144, Unité associée à l'Université Pierre et Marie Curie, Gif-sur-Yvette, France
[b] Medical Center for Molecular Biology, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia
[c] Department of Pathology, Institute of Oncology, Ljubljana, Slovenia
[d] CNRS, Laboratoire d'Ingénierie des Systèmes Macromoléculaires, UPR9027, GDR2352, Marseille, France

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ABSTRACT

Heavy chain antibodies are naturally occurring in *camelidae* (camels and llamas). Their variable domain (VHH) can be efficiently produced as a recombinant protein in *E. coli* with a large range of applications in the fields of diagnostics and immunotherapy. Standard cloning approach involves resolution of VHH from the heavy chain variable domain of conventional antibodies (VH) by a nested PCR amplification followed by a phage display based selection. Present work illustrates that in contrast to usual finding, specific, good affinity and efficiently expressed VH domain of conventional antibodies can be selected from the co-amplification products of VH and VHH cDNAs. Sequence analysis illustrated that following the two first rounds of selection against cancer markers, similar number of VH and VHH binders were observed. However, after a third round, the more specific binders directed against p53, VEGF, BCL-2 proteins surprisingly contain only VH specific hallmarks. Characterisation of the specificity, affinity and productivity of selected VH binders is described. Because llama VHs show higher sequence and structural homology with the human VH III group than llama VHHs (Vu et al., 1997), they constitute very interesting agents in therapeutic applications, especially in human immunotherapy and cancer treatment.

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1. Introduction

Heavy chain antibodies (HcAb) naturally occur in *camelidae* (camels and llamas). In their serum conventional heterodimer antibodies and homodimer HcAb coexist (Hamers-Casterman et al., 1993) in relative amounts depending on species (van der Linden et al., 2000). In HcAb, the light chain and first constant domain (CH1) are absent and their variable domains, referred to as VHH (Arbabi Ghahroudi et al., 1997), constitute the smallest (about 120 amino acids) naturally occurring fragment capable of binding an antigen. VHH can be obtained from *E. coli* (Olichon and Surrey, 2007), *S. cerevisiae* (Frenken et al., 2000), *Lactobacillus* (Hultberg et al., 2007), *A. awamori* (Joosten et al., 2005), *P. pastoris* (Rahbarizadeh et al., 2006) and tobacco plant (Rajabi-Memari et al., 2006) with high yields. They are generally stable (Perez et al., 2001), soluble and bind specifically and with a high affinity to their targets (Desmyter et al., 2002; Hmila et al., 2008). They are able to recognise haptens (Frenken et al., 2000; Yau et al., 2003; Alvarez-Rueda et al., 2007) and cryptic epitopes, such as enzyme active site (Lauwereys et al., 1998), which are not accessible to conventional (larger) antibodies. VHHs have

Abbreviations: CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; FR, framework region; GST, glutathione S-transferase; IgG, immunoglobulin G; K_D, equilibrium dissociation constant; MPBST, milk phosphate-buffered saline-Tween 20; PCR, polymerase chain reaction; Rmax, maximum response; RT, room temperature; RU, resonance unit; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; VH, heavy chain variable domain of the conventional antibody; VHH, variable domain of a heavy chain antibody; VL, light chain variable domain of the conventional antibody.

* Corresponding author. Centre National de la Recherche Scientifique (CNRS), Centre de Génétique Moléculaire (CGM), 91190 Gif-sur-Yvette, France. Tel.: +33 1 69 82 36 80; fax: +33 1 69 82 36 82.
E-mail address: pompon@cgm.cnrs-gif.fr (D. Pompon).
[1] Present address: Institut National de la Santé et de la Recherche Médicale, Laboratoire Stress Cellulaire, U624, Marseille, France.

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been successfully used as immunotherapeutics (Harmsen et al., 2006) and to develop cancer therapeutic approaches (Cortez-Retamozo et al., 2004). They constitute promising agents in new generation of therapeutic antibodies (Behar et al., 2008). They can be used in muscular dystrophy disease treatment (Chartier et al., 2009).

VHHs are clearly distinguishable from the VHs of the conventional antibodies, by the presence of several amino acid signatures, located in the solvent-exposed surface which is normally covered by the variable domain of the light chain (VL). Using the Kabat numbering (Kabat et al., 1991), VHHs differ from VHs by the V37Y/F, G44E/Q, L45R, W47F/G/S/L substitutions on conserved VH residues belonging to the second framework (FR2) (Vu et al., 1997; Harmsen et al., 2000). The importance of these residues for the solubility was highlighted by Davies and Riechmann (1994). Expression of the VH domain from human antibodies in absence of light chain was found frequently inefficient (Worn and Pluckthun, 1998) because of aggregation, poor-solubility and stickiness. This led to the usual finding that the llama VHs are similarly not suitable for high efficiency expression. This finding was supported by Davies and Riechmann (1996) who reported that substitution of the four amino acids in human VH aligning with the hallmarks of camelid VHH allowed to improve the solubility of the expressed domain and reduce aggregation. Until now, soluble llama conventional VHs were isolated by Tanha et al (2002), but only a single structure of llama VH was resolved by NMR (PBD code-1IEH) (Vranken et al., 2002).

Based on these previous findings, standard methods for llama VHH library construction were designed to avoid VH contamination in VHH libraries. Following mRNA isolation and CH2 gene specific reverse transcription, usually two successive PCRs are performed. The first is used to discriminate VH from VHH based on amplicon sizes which differ by ~300 bp in length. Shorter amplicons (620 and 690 bp) encoding VHHs are subsequently re-amplified through a second nested PCR with primers annealing at the codons of FR1 and FR4, because all variable domains of camelid heavy chain antibodies belong to a single family (family III). Afterwards VHH fragments are cloned and displayed for selection. Such a nested PCR approach could be mutagenic and disadvantageous for molecular diversity, but is expected to eliminate VH derived contributors which could lead to sticky proteins through exposed hydrophobic amino acids on their surface lacking VL domain.

In contrast to described VHH advantages, llama VHs show a higher similarity with the human VH III family than VHH (Vu et al., 1997). Because of this better similarity, they are expected to be less immunogenic in humans and could represent a significant advantage for immunotherapy and other therapeutic applications. Here we describe a single step PCR method to construct a llama VH–VHH mixed library and an adapted selection procedure. This work illustrates on several examples (cancer protein markers p53, BCL-2 and VEGF), that VH good affinity binders can be easily selected and efficiently produced in *E. coli*. Analysis of the sequences at different stages of the selection process is presented as well as the specificity, affinity and expression of selected binders. Results are discussed on the basis of predicted structural features.

2. Materials and methods

2.1. Llama antibody library construction and characterisation

Adult female llama (*Lama glama*) was immunised by a single sub-coetaneous injection of human protein extract followed by three booster injections every 3 weeks. Protein extract was prepared from post-operative samples of six patients suffering from different types and stages of gastric cancer. Native proteins were extracted using liquid nitrogen homogenization and standard biochemical techniques (Ha et al., 2002). Finally, extracted proteins (1.5 mg) were dissolved in PBS buffer containing 10 mM EDTA, 0.5% sodium cholate and 0.1% NP-40 detergent and mixed with an equal volume of Freund's incomplete adjuvant. The blood of immunised animals was collected and lymphocytes were separated by Ficoll-Histopaque-1077 (Sigma-Aldrich) discontinuous gradient centrifugation followed by washing with PBS, and were afterwards stored at −70 °C until further use.

Total RNA was isolated from 10^7 leucocytes by acid guanidinium thiocyanate-phenol chloroform extraction (using RNable; Eurobio, Courtaboeuf, France), verified by electrophoresis and stored at −80 °C in DEPC treated water.

Isolated total RNA was used as a template for cDNA synthesis (Super Script II™, Reverse Transcriptase, Invitrogen) using CH2 domain gene-specific primer 5′-GGTAC-GTGCTGTTGAACTGTTCC-3′, annealing at the CH2 exon of the heavy chains of all llama immunoglobulins. PCR was performed on cDNA using DyNAZYME EXT DNA Polymerase (Finnzymes) with an equimolar mixture of degenerated PAGE purified primers (Sigma Aldrich) annealing at the FR1 and FR4 of conventional (IgG1) and heavy chain antibodies (IgG2 and IgG3):

(I.) 5′-CCTATAGGCCCAGCCGGCCATGGCCGAGGTG-CAGCTGGTGSAG-3′

(II.) 5′-CCACGATTCTGCGGCCGCTGAGGAGACRGT-GACCTGGGTCC-3′

containing BglI and NotI restriction enzyme sites (underlined). PCR protocol consisted of an initial denaturation step at 94 °C for 2 min followed by 37 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 100 s, and a final step extension at 72 °C for 10 min. The resulting unique ~450 bp PCR fragment was purified from 1.5% highly pure agarose gel and digested with BglI and NotI (New England Biolabs), re-purified on gel and ligated (T4 DNA Ligase, New England Biolabs) into SfiI and NotI digested pHEN2 phagemid (between the PelB leader sequence and the M13 viral gene III). Vector comprises the β LacIq (LacZ) promoter, a pelB leader sequence, C-terminal myc-tag (EQKLI-SEEDLN) and $(His)_6$ tag to facilitate purification, amber stop codon and bacterial phage M13-derived g3 gene. After electrotransformation into TG1 *E. coli* (Stratagene, USA) the cells were plated on selective plates. Library size was calculated by plating serial dilution aliquots on 2YT/ampicillin (100 μg/ml) containing 2% glucose (2YTAG) agar plates and incubated overnight at 30 °C. Colonies were scraped from the plates with liquid 2YTAG and library was stored at −80 °C in the presence of 20% glycerol. Fifty clones were sequenced (GATC company) for diversity analysis using 5′-M13 reverse and 3′-M13 forward universal primers. All *in situ* PCR reactions were performed using Taq DNA polymerase (New England Biolabs).

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