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Streamlined method for parallel identification of single domain antibodies to membrane receptors on whole cells

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ABSTRACT

Background: Owing to their minimal size, high production yield, versatility and robustness, the recombinant variable domains (nanobodies) of camelid single chain antibodies are valued affinity reagents for research, diagnostic, and therapeutic applications. While their preparation against purified antigens is straightforward, the generation of nanobodies to difficult targets such as multi-pass or complex membrane cell receptors remains challenging. Here we devised a platform for high throughput identification of nanobodies to cell receptor based on the use of a biotin handle.
Methods: Using a biotin-acceptor peptide tag, the *in vivo* biotinylation of nanobodies in 96 well culture blocks was optimized allowing their parallel analysis by flow cytometry and ELISA, and their direct use for pull-down/MS target identification.
Results: The potential of this strategy was demonstrated by the selection and characterization of panels of nanobodies to Mac-1 (CD11b/CD18), MHC II and the mouse Ly-5 leukocyte common antigen (CD45) receptors, from a VHH library obtained from a llama immunized with mouse bone marrow derived dendritic cells. By on and off switching of the addition of biotin, the method also allowed the epitope binning of the selected Nbs directly on cells.
Conclusions: This strategy streamlines the selection of potent nanobodies to complex antigens, and the selected nanobodies constitute ready-to-use biotinylated reagents.
General significance: This method will accelerate the discovery of nanobodies to cell membrane receptors which comprise the largest group of drug and analytical targets.

1. Introduction

The generation of potent antibodies for research, diagnosis and therapy has been facilitated by the progress in methods for building and selecting large antibody libraries [1,2]. In addition to conventional heavy/light chain antibody libraries, there has been a growing interest in single domain antibody (sdAb) libraries. These libraries derived from a special type of antibodies that occur in camelids [3] and some species of sharks [4]. These camelid heavy-chain-only antibodies are devoid of light chain and their variable domain shows a high degree of identity with the human VH3 family [5], which has been regarded as an advantageous property for their application as human therapeutic agents [6]. The sdAb antigen binding site sits entirely in the heavy chain variable domain (VHH) and thus the VHH recombinant protein, also referred as nanobody (Nb), represents the smallest antibody fragment (~15 kDa) that retains the parent functional specificity, being half the size of conventional scFv. Recombinant VHHs can be produced with high expression yields in *Escherichia coli*, as soluble and highly stable proteins, which have popularized their applications [7].

Despite the fact that the VHH antigen binding site is formed by only three and not six CDRs as in heterotetrameric antibodies, sdAbs bind their cognate antigens with similar affinity as conventional antibodies [8]. Although VHHs with specificity for a great variety of antigens have been isolated, including all types of macromolecules and haptens [7,9], the convex architecture of the sdAb paratope with an extended CDR3 appears to have evolved mainly to interact with cavities on the antigen surface. These are typically found at the active site of enzymes, and hence several sdAbs have been shown to affect the catalytic activity of enzymes [10,11]. This concave topography is also found in many cell receptors, which together with the salient properties of nanobodies make VHH libraries a very attractive source of cell-receptor agonists/antagonists.

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VHH libraries are easy to build, and in fact a comprehensive representation of the animal's original pool of specificities can be accomplished because, conversely to conventional heterodimeric libraries, there is no shuffling of the heavy and light chains [7]. However, a common scenario during the selection of these complex libraries is how to further select a particular antibody with the desired attributes from the large number of positive clones resulting from the initial enrichment. This is more challenging when the antibody target is part of a complex antigen, such as in the case of a membrane protein on the cell surface. Indeed, due to the fact that VHH antigen recognition is highly sensitive to conformational changes in the target antigen [12], the conventional approach of using recombinant membrane proteins for immunization and selection can often lead to lack of cross-recognition of the native cell receptor if proper measures to replicate its native conformation are not adopted [12]. In addition, for receptor discovery, whole cells have to be used for immunization and panning and thus there is a need for simple methods that enable the characterization of a large number of clones in a simple and systematic fashion, facilitating the identification of the cognate cell receptor even when this is unknown.

To address these limitations we optimized a methodology for the production of *in vivo* biotinylated nanobodies (BtNbs) that facilitates their characterization by ELISA, flow cytometry and pull-down experiments, which is amenable for high-throughput screening, Fig. 1. The metabolic biotinylation of nanobodies has previously been used for diagnostic applications [13,14] or for their oriented immobilization in microarrays [15]. The binding of biotin to avidins (avidin/streptavidin) in solution is regarded as one of the strongest non-covalent interactions (K_D of ~10^{-15} M). Although the conjugation of biotin through its carboxyl group is accompanied by a reduction of this affinity [16], the biotin tag provides ready and strong binding to acceptor avidins which has given rise to a profusion of avidin/streptavidin bioconjugates for countless applications [17,18]. Our labeling approach makes use of the biotin ligase BirA of *E. coli* which specifically conjugates biotin to the side chain of a Lys residue within a 15 mer acceptor peptide (BtAP) tag [19]. There is only one natural substrate of BirA, the biotin carboxyl carrier protein (BCCP) of *E. coli* is a minor component of the bacterial cell extract [20] and does not interfere with the intended use of the BtNb. Besides facilitating the isolation of nanobodies against complex targets, the selected antibodies can straightaway be produced in large amounts as biotin-labeled ready-to-use reagents.

2. Methods

2.1. Construction of the pINQ-BtH6 vector

A triclocarban (TCC) specific VHH (T7) cloned between the two SfiI sites of the pComb3 vector [21] was used as template for PCR amplification of the OmpA-SfiI(1)–VHH-SfiI(2) region using the forward aatatctagaaataattttgtttaactttaagaaggagatataccatgaaaaagacag ctatcgcgattg and reverse atttctcgagttcgtgccattcgattttctgagcctcgaagat gtcgttcagaccgccaccttggccggcctggcctgaggagacg primers. Upstream of the annealing sequence, the forward primer contained the ribosomal binding site (rbs) of the pET 28a(+) vector (Noavagen) including the XbaI restriction site (underlined). Similarly, the 5′ of the reverse primer contained the coding sequence for the peptide BtAP and the XhoI restriction site (underline). To assemble the pINQ-BtH6 vector, the amplicon was digested with XbaI and XhoI and cloned into the pET 28a(+). The cloning/expression region of pINQ-BtH6 is shown in Fig. S-2 (Supporting information).

2.2. In vivo biotinylation of nanobodies

The VHH genes were cloned in the pINQ-BtH6 vector using the SfiI sites and the resulting plasmid was transformed in *E. coli* BL21(DE3) (Novagen) carrying the plasmid pCY216 [22]. The transformed cells were then seeded in LB (Luria–Bertani) agar plates supplemented with 35 μg/mL of chloramphenicol and 50 μg/mL of kanamycin. Single colonies were grown in 96 deep well culture plates (grainer) in 500 μL of LB supplemented with kanamycin/chloramphenicol in the presence of 0.04% arabinose and 100 μM D-biotin at 250 rpm, 37 °C. When the OD_{600} reached 0.6 AU, IPTG was added to a final concentration of 3 μM. After 4 h the plate was centrifuged and the cell pellets were resuspended in 100 μL of PBS (phosphate buffer saline). To extract the soluble VHHs, the cells were disrupted by three cycles of freezing and thawing or with B-PER Bacterial Protein Extraction reagent (Pierce).

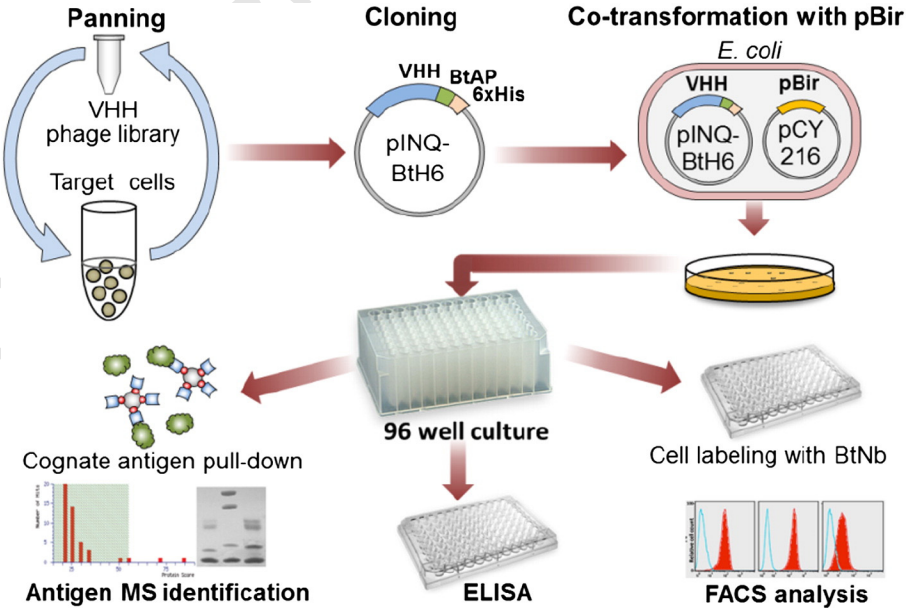

Fig. 1. Schematic diagram of the method for the isolation of biotinylated nanobodies to cell receptors. After panning on cells, the VHH output is cloned *en masse* in the expression vector pINQ-BtH6 which adds the biotin acceptor peptide. This sub-library of VHH is then co-transformed in *E. coli* together with pCY216 for over expression of the biotin ligase BirA. Individual clones are then culture in 96 deep well blocks and the soluble cell extract containing the biotinylated Nb can be characterized by different methods in a high throughput fashion.

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