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Research paper

Validation of a stable recombinant antibodies repertoire for the direct selection of functional intracellular reagents

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Abstract

We have previously generated a semi-synthetic single-chain variable fragments (scFv) phage display library built on a thermodynamically stable single-framework scaffold. All scFv antibodies selected from this repertoire showed high thermodynamic stability and were expressed as soluble molecules in bacterial cytoplasm. In this work, two complementary methodologies have been adopted to assess the functionality of library-derived scFvs as intracellular antibodies and to verify the possibility to directly use this repertoire for the selection of antibodies able to function in a reducing environment. The possibility to improve the performance of this highly stable antibody repertoire was evaluated subjecting the library to thermal denaturation and renaturation in the presence of a reducing agent before biopanning procedure. The scFv clones obtained after this treatment resulted the same isolated using standard biopanning conditions, suggesting that the selection efficiency of this repertoire is not affected by disulphide bonds formation. This evidence was confirmed by surface plasmon resonance analysis, measuring antigen affinity of a panel of library-derived scFv fragments both in oxidizing and reducing conditions. We observed perfectly comparable rate constants for antigen–scFv interactions in both antibody redox formats, demonstrating complete functionality also in the absence of intra-domain disulphide bonds. The experimental data point out that it is possible to straightforwardly isolate from this library scFvs with different specificities able to be functionally expressed in the cell cytoplasm. Hence, this library represents a valuable source of intrabodies for therapeutic applications.

Keywords: Phage display; scFv; Intrabody; Surface plasmon resonance

1. Introduction

Antibodies represent ideal reagents for a wide range of applications due to their capacity to bind molecules with high affinity and specificity. The recombinant antibody technology allowed to engineer the complex structure of an immunoglobulin in different formats, the most effective being the single-chain Fv (scFv). In this format the heavy and light chain antibody variable domains (V_H and V_L respectively) are connected by a flexible linker, resulting in a smaller molecule that can be easily

Abbreviations: scFv, single-chain Fv antibody fragment; CDR, complementarity determining region; V_H, heavy chain of an antibody Fv fragment; V_L, light chain of an antibody Fv fragment; PVX, potato virus X; DTT, dithiotreitol; IPTG, isopropyl-β-D-galactopyranoside; PBS, phosphate buffer saline; GST, glutathione *S*-transferase; ELISA, enzyme-linked immunosorbent assay; CMV, cucumber mosaic virus; HEL, hen egg lysozyme; redox, oxidation-reduction; HRP, horseradish peroxidase.

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expressed in different heterologous systems: bacteria, yeasts, plant and animal cells (Kipriyanov and Le Gall, 2004). The proper folding of a scFv fragment depends on the intra-chain disulphide bonds that stabilize the molecule scaffold. However, many applications require the expression of functional antibody fragments in cellular compartments, such as the reducing milieu of the cytoplasm, that do not allow efficient disulphide bond formation. Only few intrinsically stable intracellular antibodies ("intrabody") can be expressed in this cell compartment to bind to cellular targets, neutralizing or modifying their function or localization. Such molecules represent promising tools not only for disease therapy (Lobato and Rabbitts, 2004; Stocks, 2005; Williams and Zhu, 2006), but also for basic research (i.e. to study basic biological processes associated to functional genomic approaches) (Huston and Gorge, 2001; Visintin et al., 2004).

Conventional approaches, based on the assembly of V_H and V_L domains isolated from hybridomas or on the selection from phage display libraries, do not always guarantee the isolation of functional intrabodies. Several strategies have been adopted to obtain scFv fragments able to bind the antigen also in the cytoplasm milieu. Most of these approaches are based on the screening of repertoires using stringent conditions that allow the selection of stable antibodies able to function in a reducing environment. Examples of such methodologies are based on the presence of a reducing agent in the culture medium to isolate cell expressing scFv mutants (Martineau and Betton, 1999) or on the selection of scFv–antigen pairs interacting in yeast cytoplasm ('Two-Hybrid Technology') (Visintin et al., 1999; Auf der Maur et al., 2002) and in human cells ('Direct Phage to Intrabody Screening') (Gennari et al., 2004). Alternatively, it is possible to improve antibody performances by rational design (Donini et al., 2003; Ohage and Steipe, 1999; Wirtz and Steipe, 1999) or molecular evolution applied on the scaffold (Proba et al., 1998; Martineau et al., 1998; Jermutus et al., 2001). Functional intrabodies have been obtained using these approaches that often require laborious experimental procedures and/or specific knowledge of the structure. Additionally, the cytoplasmic expression of scFv antibodies has been improved by fusion to carrier proteins, such as maltose binding protein (Bach et al., 2001) or thioredoxin (Jurado et al., 2006), but the outcome is not always predictable and depends essentially on the biological system. The possibility to modify *E. coli* strains in order to promote the formation of disulphide bonds of cytoplasm-directed antibodies is also reported (Proba et al., 1995; Venturi et al., 2002; Jurado et al., 2002).

Antibody libraries could be a convenient way to speed up the isolation of intrabodies. In this view, to overcome transformation efficiency limits intrinsic to the yeast two-hybrid system, the 'intracellular antibody capture technology' (IACT) was developed. With this methodology a pool of recombinant antibodies specific for the antigen of interest is selected from a complex phage display library, followed by two-hybrid screening (Visintin et al., 2002; Tse et al., 2002; Tanaka and Rabbitts, 2003). Recently, the 'protein fragment complementation assay' (PCA) was proposed, demonstrating the possibility to screen combinatorial libraries in *E. coli* and to isolate antigen–antibody pairs interacting in the cytoplasm through the reconstitution of a bacterial growing factor (Koch et al., 2006).

The use of highly stable scaffolds for phage display libraries construction has been already reported (Auf der Maur et al., 2002; Tanaka and Rabbitts, 2003), but the selection of intrabodies from such repertoires is restricted to a further *in vivo* antigen–antibody interaction step. Although the *in vivo* selection-based methods guarantee the isolation of intracellular antibodies, they often show limits such as low selection efficiency (due to unspecific intracellular antibody-antigen interaction events) or the isolation of low affinity binders.

The success of the strategies for intrabody selection based on phage display library essentially depends on scaffold stability. We demonstrated that a superior intrabody scaffold endowed with high stability (Tavladoraki et al., 1999) can be used to construct a phage display repertoire consisting of scFv fragments with the same intracellular stability of the cognate antibody. We have already established that from such repertoire, named 'F8 library', it is possible to select highly stable antibodies (Desiderio et al., 2001). In the present work, we provide evidence that all analyzed 'F8 library' constituents are also able to recognize the antigen in reducing environment. Therefore, this repertoire can be used to isolate, through the conventional biopanning protocols (Nissim et al., 1994), scFv fragments ready for intracellular expression without any further antigen-binding *in vivo* procedure.

2. Materials and methods

2.1. Panning in stringent conditions

To evaluate the stability of phage particles after heat treatment, aliquots of 200 μl (corresponding to about 10^{12} phage particles) were warmed at 60 °C for 1 h and slowly cooled at room temperature. Phage suspension was then used to infect 10 ml of an *E. coli* TG1 strain culture in exponential growth ($OD_{600\ nm}$=0.5–0.7), by incubating