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Intrabodies – Valuable Tools for Target Validation

Selection Procedures for the Use of Intrabodies in Functional Genomics

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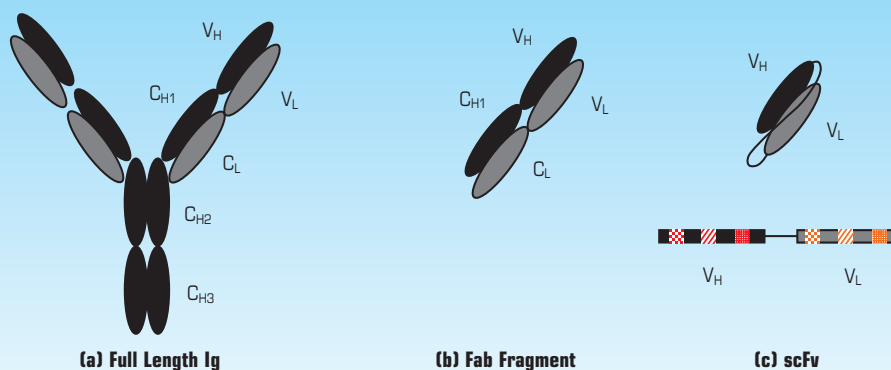
As more and more genomes become sequenced, the number of novel proteins with unknown function is steadily increasing. Consequently, a considerable interest exists to characterise the function of many gene products and to validate them as potential drug targets in particular disease pathways. Both gene knockout and antisense techniques are employed for this purpose, but they have their limitations. Intrabodies present an attractive alternative to modulate protein function and analyse the effects. Moreover, they can also be employed to target specific domains of the protein. However, the stability of an intrabody is often greatly impaired by the reducing intracellular conditions in the cell that prevent the formation of disulfide bridges. In this article we review a number of examples for the intracellular use of antibodies and discuss selection procedures for the identification of functional intrabodies.

Antibodies have long come of age. Playing their natural role in the immune system by defending the organism against pathogens, they are now universally employed for therapeutic and diagnostic applications and research. Often they are also used as recombinant proteins with little resemblance to the original molecule other than its ability to specifically recognise and bind a target protein with high affinity. The domain

structure of antibodies allows the engineering of antibody fragments in many different forms, the selection of the desired functions (that is, specificity for the antigen and immunogenic properties), and even combining with heterologous effector domains.

Recombinant antibody technologies also permit the generation of antibodies directed against

Figure 1: Schematic Representation of an Antibody and Recombinant Fragments



- a) Naturally occurring full-length immunoglobulin; MW: ~150kDa
 b) Recombinant fab fragment, lacking the C_{H2} and C_{H3} domain, which is responsible for the effector functions of the Ig; MW: ~60kDa
 c) Single-chain Fv fragment, containing the variable domain of the heavy and the light chain, connected by a linker peptide, MW: 20-30 kDa

highly conserved proteins or self-antigens, which were hitherto difficult to obtain. The generation of specific high-affinity antibodies by conventional methods, such as repeated animal immunisation or hybridoma technology, will therefore increasingly be replaced by selection from recombinant antibody libraries. The recombinant technologies are faster and cheaper, while antibodies of comparable affinities can be isolated. The process of selection involves a number of different procedures that all have their advantages and limitations. The choice of method depends on the final application of the antibody fragment and the requirements the antibody has to fulfil.

The minimum antibody form still retaining specificity and monovalent binding affinity of the full-size parent antibody, is the single-chain Fv fragment (scFv). It contains the variable region of the heavy and the light chain, linked by a flexible linker and expressed from a single gene. The variable domains of the heavy and the light chain contain the complementarity-determining regions (CDRs) of the parent antibody – the main antigen binding domains – which determine the specificity of the scFv (see Figure 1).

INTRABODIES AND THEIR MODE OF ACTION

Intrabodies are scFvs that are expressed within the cell and directed against intracellular proteins. In this way they can interfere and inhibit cellular processes inside the cell in a number of ways, affording them great potential for the use in target validation. Intrabodies can inhibit an enzymatic activity directly, or interfere with protein-protein interactions, thus disrupting signalling pathways. They can also be used to displace a protein from its site of action. The fusion of intracellular localisation signals, such as a nuclear localisation signal (NLS) or a retention signal for the endoplasmic reticulum (ER), can be used to re-direct the antibody and its target antigen to specific locations within the cell. For instance, an scFv directed against the ErbB-2 receptor and designed to prevent transit through the ER was shown to down-regulate the surface expression of ErbB-2 and consequently, to considerably affect growth factor signalling (1).

Another elucidating study on the action of intrabodies was described by Lener *et al* (2). The authors compared the inhibition of ras function with a number of different scFv fragments *in vivo* and *in vitro*. Their ability to inhibit ras function was then characterised *in vivo* using a cell proliferation assay and *in vitro* measuring

GTPase activity. Although none of the selected scFvs were able to inhibit ras function *in vitro*, it emerged that a number of them still functionally inhibited ras *in vivo* by diverting it from the plasma membrane and sequestering it in the nucleus or by the formation of aggregates. These studies illustrate the potential of intrabodies to validate targets in cellular assays.

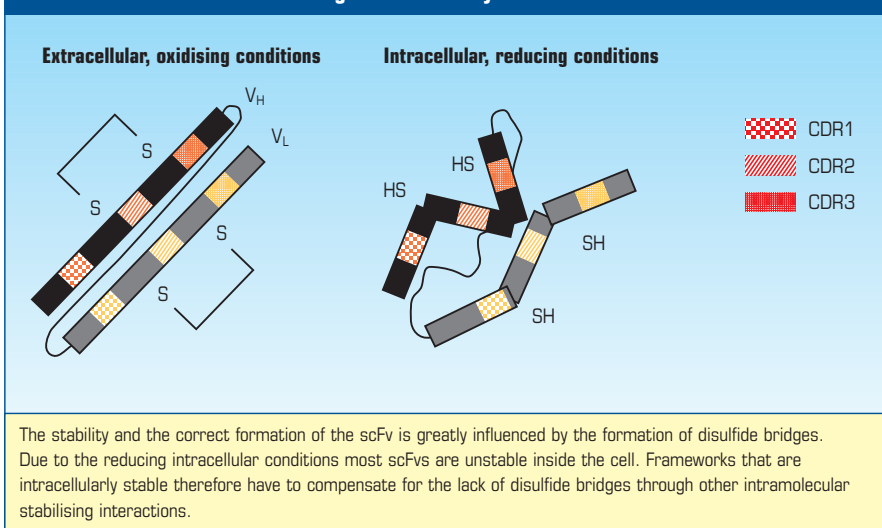
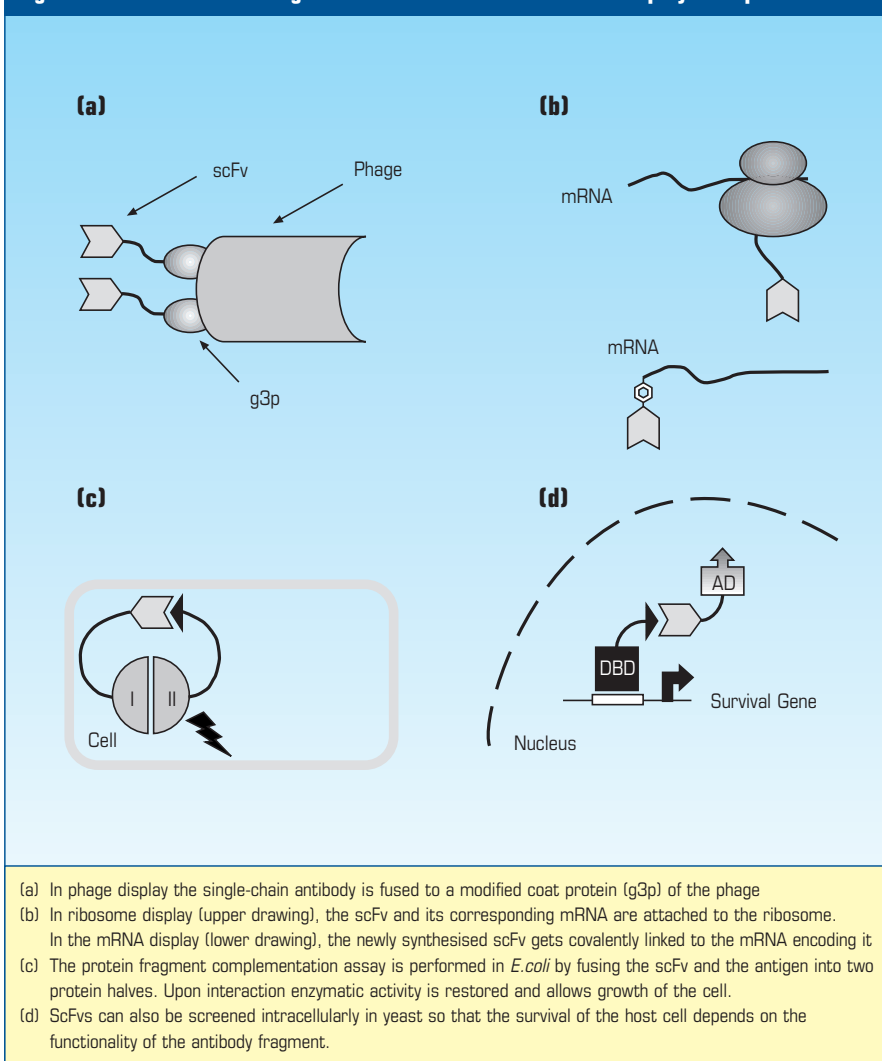
POTENTIAL THERAPEUTIC APPLICATIONS OF INTRABODIES

A recent paper (3) describes the use of human scFvs directed against huntingtin protein to interfere with the formation of intracellular aggregates characteristic of Huntington's disease. The inhibition of protein aggregation required the fusion of a nuclear localisation signal (NLS) to the scFv, which was subsequently able to retarget the huntingtin derivative to the nucleus. The authors suggest that the binding event, by maintaining huntingtin protein in a soluble state, favours normal cellular protein turnover rather than aggregation (3). This 'solubilising' property of scFvs could be interesting for a range of neuro-degenerative diseases caused by abnormal protein aggregation.

A further field of application for scFvs is the therapy of HIV. The scFv 105 against the HIV Env protein (gp120) was shown to inhibit the proteolytic processing of the precursor protein gp160 in the ER and decrease the infectivity of HIV virions released by the cells (4). Another protein essential for the life cycle of HIV that has been targeted by various scFvs is Tat, which is required for the transactivation of the HIV-LTR (5). An scFv targeted to Tat blocked the latter's nuclear function. Interestingly, nuclear targeting of the scFv was not required for this effect. This suggests that the scFv-mediated effects were exerted by sequestering Tat in the cytoplasm, rather than interfering mechanistically with its nuclear function. Similarly, scFvs directed against HIV Rev were shown to prevent the cytoplasmic nuclear shuttling of Rev, a regulatory RNA binding protein. Interestingly, the *in vitro* affinities of the scFvs did not always correlate with the observed performance in the cellular system – some scFvs with lower affinity displayed a more potent inhibitory effect *in vivo*. This demonstrates the importance of considering the different requirements of the intracellular environment for the functionality of an antibody.

Finally, scFvs have been successfully employed in plants, protecting them against viral infections through a quasi-intracellular immunisation. The expression of a functional scFv against artichoke

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Figure 2: Stability of scFvs**Figure 3: Schematic Drawing of the Multifaceted Formats to Display or Express an scFv**

mottled crinkle virus (AMCV) in tobacco plant cells, resulted in a significant reduction of infection and a delay in symptoms (6). Expression of scFvs is therefore considered to be a promising approach to increase resistance of plants to viral diseases. The examples described above highlight the potential of intrabodies for target validation and possibly even therapeutic use in the long-term.

Despite these encouraging results, the use of antibodies inside the cell is fraught with difficulties regarding stability and solubility. This has limited their widespread use to date. In their natural function antibodies are secreted from the cell and operate in the extra-cellular oxidising environment of the bloodstream. This allows the formation of disulfide bridges that contribute greatly to the stability of the antibody. In addition to the inter-molecular disulfide bridges important for the assembly of the full size antibody, intra-molecular disulfide bridges are important for the structural integrity of the antibody, and also the scFv. Therefore, only a small proportion of antibodies are functional under the reducing intracellular conditions, while the majority of antibody frameworks become unstable, insoluble and therefore non-functional (see Figure 2). However, as the potential of antibodies for applications inside the cell is becoming increasingly recognised, a number of selection procedures have been developed to isolate functional intrabodies. Further development of these technologies will greatly facilitate the exploitation of intrabodies.

SELECTION METHODS FOR scFvs AND INTRABODIES

Phage Display

The phage display technology has been successfully employed for the isolation of new scFvs from libraries (7). To select scFvs binding to a particular antigen, the scFvs are fused to a coat protein, typically pIII (g3p) of filamentous M13 phage (see Figure 3a). An scFv on the phage that binds an immobilised antigen is enriched during consecutive cycles of binding, elution, and amplification after infection of bacteria – a process called panning. However, enrichment of scFv-displaying phages is often hampered by concomitant enrichment of false positives, particular when harsh elution conditions are employed to isolate high affinity binders. In practice, the optimal conditions for selection of scFvs have to be determined for each new antigen. For the isolation of scFvs that are also intracellularly stable, a reducing agent in the elution buffer has been employed. However, it is unclear whether these conditions alone reproduce the intracellular environment adequately.

Ribosome and mRNA Display

Ribosome display (8) is an *in vitro* method that links the peptide directly to the genetic information (mRNA). An scFv cDNA library is expressed *in vitro* using a transcription-translation system. The translated scFvs are stalled to the ribosome linked to the encoding

mRNA (see Figure 3c, p76). The scFv is then bound to the immobilised antigen and unspecific ribosome complexes are removed by extensive washes. The remaining complexes are eluted and the RNA is isolated, reverse transcribed to cDNA and subsequently re-amplified by PCR. The PCR product is then used for the next cycle of enrichment. A variation of the ribosome display is mRNA display, where the mRNA becomes directly attached by a stable covalent linkage to the peptide or protein it encodes (9). The advantage of ribosome and mRNA display lies in the complexity of the scFv library that can reach up to 10^{14} different molecules. Ribosome display can be even further diversified during the PCR steps using low-fidelity polymerases. Thus, high-affinity antibodies initially not present in the libraries can be generated and selected. However, this technology offers no means of specifically selecting intracellularly stable scFvs.

Protein Fragment Complementation Assay (PCA)

This is a cellular selection procedure based on the complementation of a mutant dihydrofolate reductase (DHFR) in *E.coli* by the mouse protein (mDHFR) (10). The murine DHFR is dissected into two parts, which are expressed as fusion proteins with potentially interacting peptides. The interaction of the fusion proteins restores the enzymatic activity of mDHFR, and thus bacterial proliferation. A recent study to screen scFvs indicated that only a specific interaction of antibody and antigen allows the functional complementation of DHFR (11), which makes the system amenable for the selection of intracellular scFvs. A major advantage of this system (over phage display, for example) is that the antigens do not have to be expressed, purified, and immobilised. Since the antibodies are expressed in an intracellular environment, they are dependent on a framework that is stable under these conditions. However, prokaryotic cells differ from eukaryotic cells, which are usually the desired cellular systems for intrabodies. Therefore, a need exists to reliably select stable and soluble scFvs for use as intrabodies in eukaryotic cells.

SELECTION FOR INTRACELLULAR STABILITY AND AFFINITY IN YEAST

Yeast is a well-characterised eukaryotic cellular model system that is easily manipulated. It is very valuable for screenings, due to the ease of isolation of genes with desired functions from colonies derived from a single cell. It

therefore also lends itself to the selection of functional scFvs *in vivo* in a eukaryotic cellular environment.

An adaptation of the yeast two-hybrid system can be employed to select for scFvs binding intracellularly to an antigen of choice. The scFvs are fused to a transcriptional activation domain and the antigen is expressed in fusion to a DNA binding domain. The stable interaction between the scFv and the antigen leads to the activation of the transcriptional complex and expression of a reporter gene, essential for growth of the yeast cells. Cells where the interaction fails to take place are unable to grow, which allows the identification of functional binders from the yeast colony that derives from the cell containing the original clone (12) (Auf der Maur *et al*, manuscript in preparation).

Since the intracellular stability and solubility presents a major bottleneck for the application of intrabodies as modulators of protein function, a selection procedure based on intracellular functionality and stability, rather than on antigen binding only, has been developed. In this system, cellular proliferation in the first instance depends solely on the presence of a stable and soluble single-chain antibody inside the cell. It was shown by denaturation experiments that the solubility and functionality *in vivo* correlates with the stability *in vitro* (13). This approach, named 'quality control', allows the selection of stable intrabodies independent of their binding specificities (14). With this approach it is possible to isolate 'super-stable frameworks', optimised for intracellular applications. This method also allows the selection of highly stable antibodies for a variety of other applications, such as the improvement of existing antibodies employed for diagnostic purposes.

Super-stable frameworks can also be used as acceptors for randomised CDR libraries. Although the CDR can contribute to the overall stability of the scFv, a highly stable framework will tolerate the exchange of CDR sequences in most cases. These scFv libraries in stable frameworks can then be used to isolate scFvs with new affinities and specificities using the aforementioned interaction screening in yeast.

A major bottleneck for the screening of large scFv libraries in yeast is the comparatively low transformation efficiencies that can be achieved in this organism. However, it is possible to select binders from less complex libraries and subsequently improve the affinity by further randomisations of CDR and residues

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known to be important in stability and functionality of the antibody.

Yeast has further advantages for the use in functional genomics. Since the molecular components of many disease pathways can be easily reconstituted in yeast, the system can be employed to further validate the scFv, not purely on the basis of antigen binding, but also with regard to its ability to disrupt or interfere with a molecular interaction of interest.

The potential of intrabodies as a versatile tool for target validation is becoming increasingly apparent. Selection procedures for specifically binding scFvs have been developed and some take the particular requirements in respect to intracellular stability into account. ♦

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