[Frontiers in Bioscience 12, 1344-1352, January 1, 2007]

Protein *i*: interference at protein level by intrabodies

Jayapal Manikandan, Peter N Pushparaj, and Alirio J Melendez

Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

TABLE OF CONTENTS

1. ABSTRACT

Immunoglobulin molecules have long been used in biomedical research as *in vitro* tools for identification, purification and functional manipulation of target proteins. The specificity and diversity of immunoglobulins can be exploited to target a wide range of intracellular proteins by expressing them *in vivo*. Such antibody molecules, which are expressed intracellularly and directed to defined sub-cellular compartments, are termed 'intrabodies'. They represent a new and versatile tool that has the potential to manipulate diverse biological processes. Gene knockout, antisense and small interfering RNA knockdown techniques, are employed to characterize the function of many gene products and to validate these gene products as potential drug targets for novel therapeutics, but they have their limitations. The experimental results to date suggest that intrabodies represent a powerful alternative to modulate protein function and analyze its effects. Moreover, they can also be utilized to target specific domains of a particular target protein. Even though their clinical application might take a long time, they can play an important role for target identification and validation in functional genomics and/or proteomics. Here we review the latest advances in the concept, construction and applications of Intrabodies.

2. INTRODUCTION

Immunoglobulins play a key role in the immune system of vertebrates by specifically recognizing the target antigens and binding to them with high affinity. They exhibit extreme diversity within each individual. Antibodies are secreted by differentiated B lymphocytes in response to a particular antigen. The specific and high affinity binding properties of immunoglobulin molecules has long been used in biomedical research as an *in vitro* tool for identification, purification or functional manipulation of target proteins (1). Moreover, the explosive amount of data coming up from recent high-throughput genomic and proteomic methodologies calls for the development of novel tools for functional genomics; a challenge at the moment is to develop a general technique to interfere with protein-protein or protein-DNA interactions in complex regulatory and signaling networks (2). The specificity and diversity of immunoglobulins can be exploited to target a wide range of intracellular proteins by expressing them *in vivo*. Such antibody molecules expressed intracellularly and directed to defined sub-cellular compartments, are termed 'Intrabodies' (3). The concept of using intrabodies against a specific target is based on the induction of a phenotypic knockout of the target molecule, either by directly inhibiting the function of

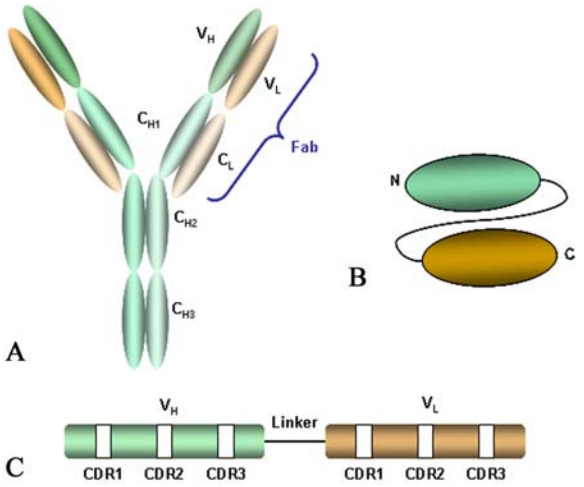

Figure 1. Schematic representation of components of immunoglobulin molecule and stable format for an Intrabody. A. Antibodies (MW~150kDa) comprise two identical heavy (H) chains (green) and two identical light chains (orange). The L chains possess one constant (C) domain and the H chain possesses three. Recombinant fab fragment (MW: ~60kDa) is responsible for the effector functions of the Ig, which lacks the CH3 and CH2 domain. B. The single-chain Fv (scFv) antibody fragment consists of a VH (green) and a VL (orange) domain, connected by a flexible linker peptide. The N-terminal (N) parts of both domains are responsible for binding antigen. (c) Linear depiction of an scFv with a short linker between the VH and VL segments. The three CDRs of each domain are the hypervariable sequences that are the key contributors to antibody diversity.

the target, or by diverting it from its normal intracellular location (4). In other words, if the antibody chains or domains are equipped with suitable localization signals, it can be targeted towards a new ectopic intracellular site to interfere with endogenous targets. This approach exploits the specificity of antibodies produced by the cellular machinery to selectively block an intracellular target. This review summarizes the recent advances in concept, construction and applications of Intrabodies.

3. INTRACELLULAR MODES OF ACTION

Intrabodies are scFvs expressed within the cell and directed against intracellular proteins. In this way they can interfere and inhibit cellular processes inside the cell by inhibiting the action of key molecules, thus providing great potential for their use in target validation and as therapeutics. With high affinity and specific binding properties, intrabodies can modulate cellular physiology and metabolism by a wide variety of mechanisms. Intrabodies have been used to block protein-protein or protein-nucleic acid interactions (5); to directly promote cell death by inducing apoptosis-related proteolytic cascades (6-8); or, albeit indirectly, to facilitate selective degradation of target proteins by recruitment to the ubiquitin-proteasome pathway (9). In a recent study, by targeting the enzymatic activity of an intracellular kinase (eg Etk) with an intrabody, it was shown that intrabodies could modulate the role a kinase plays in cell transformation in an extremely specific manner. Another interesting study on the action of intrabodies was described by Lener *et al* (10). Here the authors compared the inhibition of ras function with a number of different scFv fragments *in vivo* and *in vitro*. Although none of the selected scFvs were able to inhibit ras function *in vitro*, it emerged that a number of them did inhibit ras function *in vivo,* by diverting it from the plasma membrane and sequestering it in the nucleus, or by the formation of immune complexes. These studies illustrate the potential of intrabodies to validate targets in cellular assays. Table.1 shows the modes by which intrabodies affect intracellular protein function.

4. METHODS OF MAKING AND SELECTING INTRABODIES

The method 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 fulfill. Intrabodies are usually single chain variable fragments (scFv) comprising a heavy (H) and a light (L) chain variable (V) domain, held together by a flexible linker polypeptide to create a single polypeptide chain (11-14) (Figure 1). 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.

A straightforward approach to intrabody isolation is by their derivation from the V regions of a high-affinity monoclonal antibody (mAb) (3) (Figure 2a). The VH and VL can be amplified by reverse transcriptase (RT)–PCR of mRNA from the original hybridoma and cloned as a scFv (15, 16). Alternatively, *in vitro* display systems such as phage display, ribosome and mRNA display and yeast two-hybrid system can be employed to screen scFv libraries with the desired target, in order to select specific scFvs, which can subsequently be tested as Intrabodies (17).

4.1. Phage display

Phage display technology has been successfully employed for the isolation of new scFvs from libraries (18). To select scFvs binding to a particular target, the scFvs are fused to a coat protein, typically pIII (g3p) of filamentous M13 phage (Figure 2b). An scFv on the phage that binds an immobilised target molecule is enriched during repeated cycles of binding, extraction, and amplification after infection of bacteria. In practice, the optimal conditions for selection of scFvs have to be determined for each new target.

4.2. Ribosome and mRNA display

Ribosome display is an *in vitro* method that links the peptide directly to the mRNA (19). A scFv cDNA library is expressed *in vitro* using a transcription-translation system. The translated scFvs and its corresponding mRNA are attached to the ribosome (Figure 2c). The scFv is bound

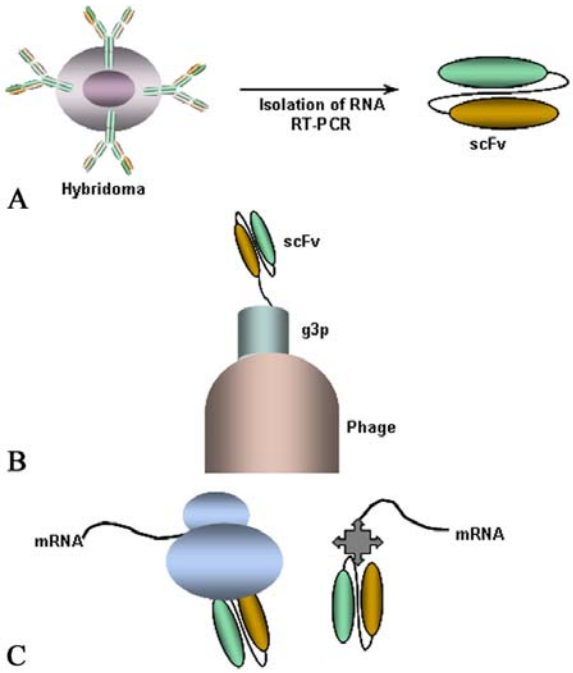

Figure 2. Methods for making and selecting intra-bodies. A. Conversion of Monoclonal antibody (mAb) to intrabody. mRNA from a hybridoma is used to amplify heavy- and light-chain variable (VH and VL) sequences by RT– PCR, which are cloned into an expression vector to make the corresponding single-chain Fv (scFv). B. In phage display the single-chain antibody is fused to a modified coat protein (g3p) of the phage. C. In ribosome display (left drawing), the scFv and its corresponding mRNA are attached to the ribosome. In the mRNA display (right drawing), the newly synthesised scFv gets covalently linked to the mRNA encoding it. D. Intracellular antibody capture technology uses *in vivo* selection to derive specific intrabodies. First, scFv phage display libraries are panned *in vitro* [as in (b)] to enrich antigen-specific scFv, and this is followed by an antigen–antibody interaction screen in yeast (which involves an antigen bait linked to the LexA DNA binding domain, which activates His3 and LacZ reporter genes after intrabody interaction with the antigen bait, because the intrabody is fused to the VP16 transcriptional activation domain) to select for the subset of scFv capable of functioning as intrabodies.

to the immobilized target, the complexes are eluted, and the RNA is isolated, then 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 (20). Although the advantage of ribosome and mRNA display is that the scFv library can reach up to 1014 different molecules, this method does not offer specific selection of intracellularly stable scFvs.

4.3. Yeast

Yeast is a well-defined eukaryotic cellular model system that is easily manipulated. It is very valuable for screenings, due to the ease of isolation of genes with preferred functions from colonies resulting from a single yeast cell. Hence, it 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 used to select for scFvs binding intracellularly to a target of choice (21). This technique also permits the selection of highly stable antibodies for a range of other applications, such as the improvement of existing antibodies employed for diagnostic purposes. In addition, super-stable frameworks can be utilized as acceptors for randomized complementarity determining regions (CDR) libraries. Even if the CDR can contribute to the overall stability of the scFv, a highly stable framework will allow 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 abovementioned interaction screening in yeast. As the molecular mechanism of several disease pathways can be easily reconstituted in yeast, the system can be employed to further validate the scFv, not only based on target binding, but also with respect to its capability of blocking a molecular interaction of interest.

4.4. Intracellular antibody capture (IAC)

Intracellular antibody capture (IAC) technology is a selection procedure for isolation of antibodies able to fold correctly and to bind target molecules under conditions of intracellular expression. It is based on the two hybrid approach, by the selection of intrabodies starting with diverse scFv phage antibody libraries, which are initially screened with antigen *in vitro,* and subsequently screening the selected scFv in a yeast *in vivo* antibody-antigen interaction assay (Fig 2d) (22,23). IAC technology identifies physically interacting antibody-target pairs. It has proved successful in allowing the selection of intrabodies which recognize a diverse set of targets, and has helped to define a scaffold of immunoglobulin V-region residues, which are particularly advantageous for intracellular function (12, 13).

Several other techniques have also been developed, which rely on a pre-determined ability of antibody fragments to fold adequately and to remain stable *in vivo* using designer scFv frameworks. It has been shown that the choice of an scFv as an intrabody should take into account its half-life and its steady-state level, to get a specific intracellular retargeting of its target molecule (7, 24-26). Moreover, multiple parameters such as intrinsic stability, monomeric or divalent forms, level of expression, affinity, and cellular localization profoundly affect the functional efficacy of intrabodies (27).

5. POTENTIAL THERAPEUTIC APPLICATIONS OF INTRABODIES

The potential of using antibodies to interfere with the biological process inside the cell in a highly specific manner can be exploited for a wide variety of applications. The significance of various modes of action of intrabodies is that specific properties of target protein can be exploited to elicit a specific response and thus to investigate or target

Table 1. Modes by which intrabodies affect intracellular protein function

S. No	Intracellular modes of action	Reference
1	Ablation of protein–protein interactions	5
2	Protein–nucleic acid interactions	5
3	Sequester a protein away from its normal site of function	10
4	Enzymes can be neutralized by binding of an intrabody	43
5	Antibody–antigen interaction-dependent apoptosis (AIDA) by activating caspase-3 (CP3).	23
6	Linking an intrabody to an F-box protein, leading to ubiquitination and subsequent degradation by the proteasome	9

Table 2. Various types of intrabodies used for therapeutic applications

Target	Intrabody format	Cellular compartment
Cancer therapy		
ATF-1/CREB	scFv	Cytoplasm
Bcl-2	scFv	Cytoplasm
BCR- ABL	scFv	Cytoplasm , ER
Caspase3	scFv	Cytoplasm
Caspase7	scFv	Cytoplasm
Cathepsin L	scFv	ER
Cyclin E	scFv-Fc	Cytoplasm
EGFR	scFv	Cytoplasm , ER
erbB2	scFv	Cytoplasm , ER
HP1	scFv	Cytoplasm
p21Ras	scFv-IgG	Cytoplasm
p53	scFv	Cytoplasm
IL-2R	scFv	ER
HIV therapy		
HIV-1 gp120	scFv, Fab	ER
HIV-1gp41	scFv	ER, trans-golgi
HIV-1RT	scFv, VH-CH 1	Cytoplasm
HIV-1Tat	scFv-C	Cytoplasm, nucleus
HIV-1Rev	scFv	Cytoplasm
HIV-1N	scFv scFv-Vpr	Cytoplasm, nucleus, virion
HIV-1p17	Fab, scFv-C	Cytoplasm
CCR5	scFv	ER
CCR4	scFv	ER
Other diseases		
Flavivirus Envelope protein	scFv	Cytoplasm
HCV NS3	scFv	Cytoplasm
Huntingtin	scFv	Cytoplasm, nucleus
IL-2R	scFv	ER
MHC-I	scFv	ER
Colicin A	scFv	Bacterial periplasm

particular protein domains (3). In contrast to most of the chemical compounds used in the clinics, intrabodies can bind to a defined intracellular target with minimum or no cross-reaction with other cellular target molecules, due to their exquisite specificity. In addition, they can be addressed to any cell compartment by adding appropriate localization sequences by molecular engineering, therefore allowing a more focused action on the targeted molecule. However, one of the major issues in the therapeutic usage of intrabodies is the efficient transfer of the target gene into the target cell. Target-cell-specific gene transfer is necessary if the intrabody targets a general pathway in normal and diseased cells. This is achieved by trans-ductional or transcriptional targeting (28, 29). If the specificity is mediated by the intrabody itself, as in the case of targeting viral proteins or over-expressed /abnormal proteins, target cell specificity is not essential, even though it might influence the therapeutic efficiency (4).

5.1. In cancer therapy

In cancer treatment, there is a wide use of antibodies, which have been selected to act on specific target proteins, expressed on the surface of the neoplastic cell after systemic or local administration. However, intrabodies can be used to target tumor specific proteins within the cell by interfering pathways associated with tumor cell proliferation, differentiation and invasion (4). Various types of intrabodies, which can be used for cancer therapy, are summarized in Table 2.

5.1.1. Inhibition or down regulation of growth factor receptors

Intrabodies against receptors such as EGFR or erbB2, IL2Rα etc have successfully been examined in different cancerous conditions. EGFR is over-expressed by a variety of tumors, including breast and ovarian carcinomas. Phenotypic knockdown of EGFR has been achieved, by retention of this receptor molecule in the ER, using intrabodies targeted to be retained in ER. This resulted in inhibition of tumor cell proliferation *in vitro* and *in vivo,* and subsequent eradication by apoptosis (30-34). The use of intrabodies to target molecules of signal transduction pathways, such as Ras protein, has been found to be valuable for tumor regression in the nude mice model of colon carcinoma (25, 26). It was shown that the p21ras oncogenic molecule is sequestered in aggregates, together with an anti-p21ras scFv derived from the Y13-259 mAb, leading to an efficient inhibition of DNA synthesis (35).

5.1.2. Intrabodies targeted at the nucleus

In metastatic melanoma, transcription factor ATF-1/CREB is highly upregulated. It has been found that the binding of ATF-1 to DNA was inhibited by the intrabody, which enabled the reduction of CRE-dependent promoter activity and suppressed tumorigenicity and metastatic potential in a melanoma cancer animal model (36, 37). Other intrabodies, like those against cyclins (e.g. anti-cyclin E intrabody), expressed as scFv–Fc fusion protein, were found to be more stable, and inhibited cell growth in breast cancer cell lines, indicating that inhibition of cell cycle regulation by intrabodies might be a potential target for cancer therapy. In another study, transcriptional activity of p^{53} mutants has been shown to revert an inactive mutant back into a functional molecule, which shows that intrabodies can restore the DNA binding. Such intrabodies may represent a novel class of molecules for p^{53} based cancer therapy (38).

5.1.3. Intrabodies targeted at extracellular proteins

Lysosomal cathepsin L, a cysteine proteinase, is found to be over-expressed and secreted by tumour cells

like melanoma cells, contributing to tumour progression. It has been shown that an intrabody directed against the procathepsin L form causes inhibition of secretion and intracellular accumulation of the protein (35). Similarly, intrabodies against other proteinases like MMP-2 and MMP-9, in ER caused down-regulation of collagenase expression and the inhibition of invasion in a highly metastatic lung carcinoma cell line (39).

5.1.4. Interference of apoptotic pathways

Caspase 3 plays the central role in apoptosis. It is converted to a tetrameric active form by upstream caspases in the pathway. But induced dimerization can cause self-activation of caspase 3 and apoptosis. It has been shown that an scFv fusion protein, containing an anti-β-galactosidase fused to caspase 3, was able to bring two caspase 3 domains in close proximity when co-expressed intracellularly with β-galactosidase (4, 23), which led to auto activation and triggered apoptosis. Proteins such as p^{53} mutants or oncogenic chimeric fusion proteins may be candidate targets for the scFv-caspase 3 approach. Potential use of scFv fusion proteins, which can activate an effector function upon scFv-mediated dimerization, opens up a new way to regulate pathways involved in cell viability or tumorigenicity. Similarly, anti caspase 7 is also a candidate for targeting apoptotic pathway in tumor cells (7). All these intrabodies are promising candidates for cancer therapy as they are tumor-specific therapeutic targets.

5.2. In treatment of HIV infection

A further field of application for scFvs is therapy for HIV. Intrabodies against HIV-1 coat proteins gp120 and gp41; also other proteins which have been developed, like matrix protein p17, reverse transcriptase (Rev), and Tat proteins, have been found to be effective in inhibiting HIV-1 multiplication in host cells. Various types of intrabodies which can be used for treatment of HIV infection are summarized in Table 2. Expression of scFv 105 against the HIV Env protein (gp120) directed to ER is 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 (40). 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 (41). An scFv targeted at 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 produced by sequestering Tat in the cytoplasm, rather than interfering mechanistically with its nuclear function. Equally promising results were obtained when scFv intrabodies against Rev proteins were used. A cytosolic intrabody against Rev can function efficiently, as Rev shuttles between the nucleus and the cytoplasm of the infected cells, whose action is essential for the nuclear export of HIV-1 structural protein mRNAs. In the case of Tat proteins, it has been found that a cytosolic intrabody, scFvTat1, directed against the N-terminal activation domain of Tat, efficiently blocked the nuclear transport of Tat and inhibited Tat mediated transactivation of HIV-1 LTR. The efficiency of scFvTat1 was significantly enhanced by the addition of the human κ light chain constant domain at the C-terminus, as it could improve the stability, although it was found to promote dimerization. Cytoplasmic or nuclear expression of intrabodies against another target HIV-1 protein, integrase, resulted in increased resistance against HIV-1 infection (5, 42-43). Another approach for HIV-1 therapy is phenotypic knockout of HIV-1 receptor or co-receptor, leading to inhibition of HIV-1 infection. Receptors like CXCR4 and CCR5 have been shown to be relevant for HIV-1 cell entry *in vivo,* have been targeted by intrabodies directed to ER, causing functional deletion or down-regulation of the receptors (44, 45).

5.3. Intrabodies for Immune suppression

Inhibition of allograft rejection, caused by the interaction between MHC-I antigens of the donor and host T cells, is essential for successful transplantations. ER-directed intrabodies against the MHC-I heavy chain were found to cause marked downregulation of surface expression of MHC-I molecules in adenovirus transduced human primary keratinocytes, even in the presence of MHC-I upregulating cytokines (46). An intrabody against the enzyme α1, 3-galactosyltransferase, to suppress the α1,3-galactosylation of the cell surface antigen; a carbohydrate recognised by xenoreactive natural antibodies in higher primates. The intrabody was able to suppress cytotoxicity mediated by anti-α1,3Gal antibodies *in vitro* by more than 90%. Though the enzymatic activity was not entirely silenced, these intrabodies may be useful to protect cells from elicited antibodies (4).

5.4. Other applications

Intrabodies may be useful for corrective treatments where expression of an altered pathogenic protein can be rectified, as in the case of polyglutamine stretches of Huntingtin protein (47-49). A recent paper reports the use of human scFvs directed against Huntington protein to interfere with the formation of intracellular aggregates characteristic of Huntington's disease (49). 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 researchers suggest that the binding event, by maintaining Huntingtin protein in a soluble state, favors normal cellular protein turnover rather than aggregation. Intrabodies have been shown to be effective in treatment of diseases like Flavi viral infections, and Hepatitis C. Apart from their therapeutic importance, intrabodies can be used for basic research as well. In one of such studies, intrabody-mediated downregulation of ErbB2 was used to demonstrate that co-expression of ErbB2 is important for signal transduction by other members of the EGFR related family (50). Table 2 summarizes the different intrabodies which can be used against the different infectious agents.

Ultimately, scFvs have been successfully applied in plants, defending them against viral infections through a quasi-intracellular immunisation. The expression of a functional scFv against artichoke mottled crinkle virus (AMCV) in tobacco plant cells resulted in a significant reduction of infection and a delay in symptoms (51). Expression of scFvs is therefore considered to be a promising approach for increasing resistance of plants to

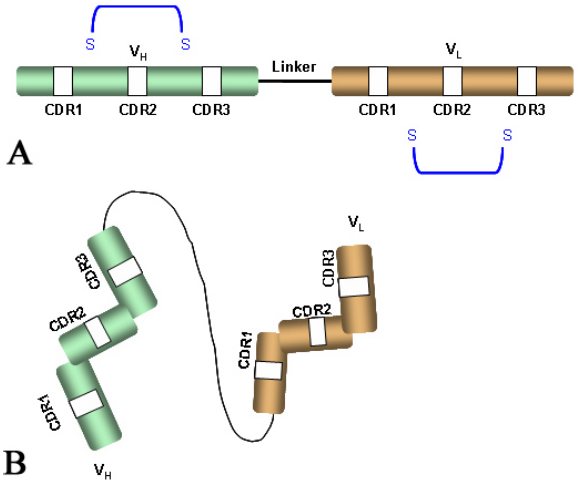

Figure 3. Schematic representation of the stability of scFvs. A. Extracellular oxidising environment of the bloodstream allows the formation of disulfide bridges that contribute greatly to the stability of the antibody. B. In the intracellular reducing environment, scFvs are unstable inside the cell.

viral diseases. The studies described above emphasize the potential of intrabodies for target validation and possibly even therapeutic use.

6. LIMITATIONS AND CHALLENGES OF INTRABODIES AS THERAPEUTIC AGENTS

Although intrabodies represent an exciting approach to treat patients with diseases where no drugs are available, there are still a number of important problems that are likely to hamper their success. The use of antibodies inside the cell is fraught with difficulties involving stability and solubility. In general, antibodies are secreted from the cell and function in the extra-cellular oxidizing environment of the bloodstream. This allows the formation of disulfide bridges that contribute greatly to the stability of the antibody (52). Due to the reducing intracellular conditions most scFvs are unstable inside the cell. Therefore, only a small proportion of antibodies are functional under the reducing intracellular conditions (Figure 3). A number of approaches have been devised to overcome the limitation imposed by the reducing cellular environment such as production of scFv fragments without disulfide bonds made by molecular evolution, stability engineering of antibody single-Chain Fv fragments etc., (53-55).

Lack of efficient gene-transfer systems that are capable of delivering the intrabody gene in sufficient numbers to the specific cell type and for maintenance of sustained expression are major hurdles to be addressed. Similarly, cloning of functional variable regions can be made difficult due to a lack of amplification of VH or VL cDNA, even when using primers optimized for RT-PCR and/or the presence of aberrant variable region transcripts, which are preferentially amplified (27). However, as the potential of antibodies for applications inside the cell is becoming increasingly recognized, a number of selection procedures have been developed to isolate functional intrabodies. Further development of these technologies will greatly facilitate the exploitation of intrabodies.

In conclusion, intracellular antibodies represent a new and versatile tool that has potential to manipulate diverse biological processes. Even though their clinical application might take a long time to achieve, they extend the application of antibodies by targeting intracellular targets which are not accessible by circulating antibody molecules. The experimental results to date suggest that intrabodies represent a powerful alternative tool to other methods of functional validation such as antisense oligonucleotides (AS-ODN) or RNAi, and could play an important role in target validation in functional genomics/proteomics.

7. PERSPECTIVES

In this review, we have provided insights into the development of protein interference by intrabodies and its future therapeutic potential in human diseases. This method exploits the specificity and diversity of immunoglobulins, in order to target a wide range of intracellular molecules by expressing them *in vivo*. The key concept of using intrabodies against a specific target is based on the induction of a phenotypic knockout of a relevant target molecule, either by directly inhibiting the function of the target molecule or by diverting it from its normal intracellular location. This approach uses the cellular machinery to produce the intrabodies, thereby harnessing the specificity of antibodies to selectively affect an intracellular target protein. Intrabodies can be used to target tumor-specific proteins intracellularly by interfering with pathways associated with tumor cell proliferation such as caspases 3, Bcl-2, p53 etc. Intrabodies can also be used to target CXCR4 and CCR5 receptors in HIV infections (41-45, 50), MHC1 in immune suppression (46), Huntington protein in Huntington's disease (48, 49) and others. However, there are some drawbacks to their success. The use of antibodies inside the cell reduces the stability and solubility. This is mainly due to the reducing intracellular conditions that most scFvs are unstable inside the cell. Therefore, only a small proportion of antibodies are functional under the reducing intracellular conditions. Hence, it might take a long time to use intrabodies clinically for therapeutic applications. However, the experimental data to-date suggests that intrabodies represent a powerful alternative tool to AS-ODN or RNAi, and could play a vital role in target validation for functional genomics/proteomics.

8. ACKNOWLEDGEMENTS

This work was supported by a grant from the NMRC/0817/2003. We thank A-K Fraser-Andrews for proofreading the manuscript.

9. REFERENCES

1. Richardson, J.H., & W.A. Marasco. Intracellular antibodies: development and therapeutic potential. *Trends in Biotechnol* 13, 306-310 (1995)

2. Visintin, M., M. Quondam, & A. Cattaneo. The intracellular antibody capture technology: towards the high-throughput selection of functional intracellular antibodies for target validation. *Methods* 34, 200-214 (2004)

3. Lobato, M.A., & T.H. Rabbitts. Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol Med* 9, 390-396 (2003)

4. Kontermann, R.E. Intrabodies as therapeutic agents. *Methods* 34, 163-170 (2004)

5. Bai, J. Sui, R.Y. Zhu, A.S. Tallarico, F. Gennar, D. Zhang, & W.A. Marasco. Inhibition of Tat-mediated transactivation and HIV-1 replication by human anti-hCyclinT1 intrabodies. *J Biol Chem* 278, 1433-1442 (2003)

6. Xu, D., J.M. Song, Y. Hu, H. Guo, D.J. Cao, P. Wang, H. Liu, & C.H. Zhao. Effects of anti-ABL tyrosine kinase intrabody on the growth of K562 cells in nude mice. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 24, 11-14 (2002)

7. Zhu, Q., C. Zeng, A. Huhalov, J. Yao, T.G. Turi, D. Danley, T. Hynes, Y. Cong, D. DiMattia, S. Kennedy, G. Daumy, E. Schaeffer, W.A. Marasco, & J.S. Huston. Extended half-life and elevated steady-state level of a single-chain Fv intrabody are critical for specific intracellular retargeting of its antigen, caspase-7. *J Immunol Methods* 231, 207-222 (1999)

8. Wheeler, Y.Y., T.E. Kute, M.C. Willingham, S.Y. Chen, & D.C. Sane. Intrabody-based strategies for inhibition of vascular endothelial growth factor receptor-2: effects on apoptosis, cell growth, and angiogenesis. *FASEB J* 17, 1733- 1735 (2003)

9. Zhou, P., R. Bogacki, L. McReynolds, & P.M. Howley. Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins. *Mol Cell* 6, 751-756 (2000)

10. Lener, M., I.R. Horn, A. Cardinale, S. Messina, U.B. Nielsen, S.M. Rybak, H.R. Hoogenboom, A. Cattaneo, & S. Biocca. Diverting a protein from its cellular location by intracellular antibodies. The case of p21Ras. *Eur J Biochem* 267, 196-205 (2000)

11. Bird, R.E., K.D. Hardman, J.W. Jacobson, S. Johnson, B.M. Kaufman, S.M. Lee, T. Lee, S.H. Pope, G.S. Riordan, & M. Whitlow. Single-chain antigen-binding proteins. *Science* 242, 423–426 (1988)

12. Tanaka, T., M.N. Loboto, & T.H. Rabbitts. Single domain Intracellular antibodies: A minimal fragment for direct in vivo selection of antigen specific intrabodies. *J Mol Biol* 331, 1109-1120 (2003)

13. Tanaka, T., & T.H. Rabbitts. Intrabodies based on intracellular capture frameworks that bind the RAS protein with high affinity and impair oncogenic transformation. *EMBO J* 22, 1025-1035 (2003)

14. Huston, J.S., D. Levinson, M. Mudgett-Hunter, M.S. Tai, J. Novotny, M.N. Margolies, R.J. Ridge, R.E. Bruccoleri, E. Haber, & R. Crea. Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single- chain Fv analogue produced in Escherichia coli. *Proc Natl Acad Sci USA* 85, 5879-5883 (1988)

15. Marasco, W.A., S. Chen, J.H. Richardson, U. Ramstedt, & S.D. Jones. Intracellular antibodies against HIV-1 envelope protein for AIDS gene therapy. *Hum Gene Ther* 9, 1627-1642 (1998)

16. Marasco, W.A., W.A. Haseltine, & S.Y. Chen. Design, intracellular expression, and activity of a human anti human immunodeficiency virus type 1 gp120 single-chain antibody. *Proc Natl Acad Sci USA* 90, 7889-7893 (1993)

17. Worn, A., & A. Pluckthun. Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 305, 989-1016 (2001)

18. Lowman, H.B., S.H. Bass, N. Simpson, & J.A. Wells. Selecting high-affinity binding proteins by monovalent phage display.*Biochemistry* 30, 832-838 (1991)

19. Hanes, J., & A. Plückthun. In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* 94, 4937-4942 (1997)

20. Roberts, R.W., & J.W. Szostak. RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci USA* 94, 12297-12302 (1997)

21. Visintin, M., E. Tse, H. Axelson, T.H. Rabbitts, & A. Cattaneo. Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proc Natl Acad Sci USA* 96, 11723-11728 (1999)

22. Tse, E., M.N. Lobato, A. Forster, T. Tanaka, G.T. Chung, & T.H. Rabbitts. Intracellular antibody capture technology: application to selection of single chain Fv recognising the BCR-ABL oncogenic protein. *J Mol Biol* 317, 85-94 (2002)

23. Tse, E., & T.H. Rabbitts. Intracellular antibody-caspase-mediated cell killing: an approach for application in cancer therapy. *Proc Natl Acad Sci USA* 97,12266-12271 (2000)

24. Cardinale, A., M. Lener, S. Messina, A. Cattaneo, & S. Biocca.. The mode of action of Y13-259 scFv fragment intracellularly expressed in mammalian cells. *FEBS Lett* 439, 197-202 (1998)

25. Cochet, O., N. Gruel, W.H. Fridman, & J.L. Teillaud. Ras and p53 intracellular targeting with recombinant single-chain Fv (scFv) fragments: a novel approach for cancer therapy? *Cancer Detect Prev* 23, 506-510 (1999)

26. Cochet, O., M. Kenigsberg, I. Delumeau et al. Intracellular expression of an antibody fragment-neutralizing p21 ras promotes tumor regression. *Cancer Res* 58, 1170-1176 (1998)

27. Bonnin, E., N. Gruel, S. Moutel, et al. Generation of functional scFv intrabodies for triggering anti-tumor immunity. *Methods* 34, 225-232 (2004)

28. Peng, K.W., & S.J. Russell. Viral vector targeting. *Curr Opin Biotechnol* 10, 454-457 (1999)

29. SchaVer, D.H., & D.A. LauVenburger. Targeted synthetic gene delivery vectors. *Curr Opin Mol Ther* 2, 155-161 (2000)

30. Beerli, R.R., W. Wels, & N.E. Hynes. Intracellular expression of single chain antibodies reverts ErbB-2 transformation. *J Biol Chem* 269, 23931-23936 (1994)

31. Deshane, J., J. Grim, S. Loechel, G.P. Siegal, R.D. Alvarez, & D.T. Curiel. Intracellular antibody against erbB-2 mediates targeted tumor cell eradication by apoptosis. *Cancer Gene Ther* 3, 89-98 (1996)

32. Deshane, J., G. Cabrera, J.E. Grim, G.P. Siegal, J. Pike, R.D. Alvarez, & D.T. Curiel. Targeted eradication of ovarian cancer mediated by intracellular expression of anti-erbB-2 single-chain antibody. *Gynecol Oncol* 59, 8-14 (1995)

33. Deshane, J., F. Loechel, R.M. Conry, G.P. Siegal, C.R. King, & D.T. Curiel. Intracellular single-chain antibody directed against erbB2 down-regulates cell surface erbB2 and exhibits a selective anti-proliferative effect in erbB2 overexpressing cancer cell lines. *Gene Ther* 1, 332-337 (1994)

34. Jannot, C.B., R.R. Beerli, S. Mason, W.J. Gullick, & N.E. Hynes. Intracellular expression of a single-chain antibody directed to the EGFR leads to growth inhibition of tumor cells. *Oncogene* 13, 275-282 (1996)

35. Guillaume-Rousselet, N., D. Jean, & R. Frade. Cloning and characterization of anti-cathepsin L single chain variable fragment whose expression inhibits procathepsin L secretion in human melanoma cells. *Biochem J* 367, 219-227 (2000)

36. Jean, D., & M. Bar-Eli. Targeting the ATF-1/CREB transcription factors by single chain Fv fragment in human melanoma: potential modality for cancer therapy. *Crit Rev Immunol* 21, 275-286 (2001)

37. Jean, D., C. Tellez, S. Huang, D.W. Davis, C.J. Bruns, D.J. McConkey, S.H. Hinrichs, & M. Bar-Eli. Inhibition of tumor growth and metastasis of human melanoma by intracellular anti-ATF-1 single chain Fv fragment. *Oncogene* 19, 2721-2730 (2000)

38. Cardinale, A., H. Filesi, S. Mettei, & S. Biocca. Intracellular targeting and functional analysis of single – chain Fv fragment in mammalian cells. *Methods* 34, 171-178 (2004)

39. Wang, W., J. Zhou, L. Xu, & Y. Zhen. Antineoplastic effect of intracellular expression of a single-chain antibody directed against type IV collagenase. *J Environ Pathol Toxicol Oncol* 19, 61-68 (2000)

40. Marasco, W.A., W.A. Haseltine & S.Y. Chen. Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. *Proc Natl Acad Sci USA* 90, 889-893 (1993)

41. Mhashilkar, A.M., J. Bagley, S.Y. Chen, A.M. Szilvay, D.G. Helland, & W.A. Marasco. Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. *Embo J* 14, 542-551 (1995)

42. Bouhamdan, M., D.S. Strayer, D. Wei, M. Mukhtar, L.X. Duan , J. Hoxie, & R.J. Pomerantz. Inhibition of HIV-1 infection by down-regulation of the CXCR4 co-receptor using an intracellular single chain variable fragment against CXCR4. *Gene Ther* 8, 408-418 (2001)

43. Levy-Mintz, P., L. Duan, H. Zhang, B. Hu, G. Dornadula, M. Zhu, J. Kulkosky, D. Bizub-Bender, A.M. Skalka, & R.J. Pomerantz. Intracellular expression of single-chain variable fragments to inhibit early stages of the viral life cycle by targeting human immunodeficiency virus type 1 integrase. *J Virol* 70, 8821-8832 (1996)

44. Steinberger, P., J. Andris-Widhopf, B. Buhler, B.E. Torbett, & C.F. Barbas.. Functional deletion of the CCR5 receptor by intracellular immunization produces cells that are refractory to CCR5-dependent HIV-1 infection and cell fusion. *Proc Natl Acad Sci USA* 97, 805-810 (2000)

45. Bouhamdan, M., J. Kulkosky, L.X. Duan, & R.J. Pomerantz. Inhibition of HIV-1 replication and infectivity by expression of a fusion protein, VPR-anti-integrase single-chain variable fragment (SFv): intravirion molecular therapies. *J Hum Virol* 3, 6-15 (2000)

46. Mhashilkar, A.M., C. Doebis, M. Seifert, et al. Intrabody-mediated phenotypic knockout of major histocompatibility complex class I expression in human and monkey cell lines and in primary human keratinocytes. *Gene Ther* 9, 307-319 (2002)

47. Colby, D.W., Y. Chu, J.P. Cassady, M. Duennwald, H. Zazulak, J.M. Webster, A. Messer, S. Lindquist, V.M. Ingram, & K.D. Wittrup. Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free single-domain intracellular antibody. *Proc Natl Acad Sci USA* 101, 17616-17621 (2004)

48. Khoshnan, A., J. Ko, & P.H. Patterson. Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation

and toxicity. *Proc Natl Acad Sci USA* 99, 1002-1007 (2002)

49. Lecerf, J.M., T.L. Shirley, Q. Zhu, A. Kazantsev, P. Amersdorfer, D.E. Housman, A. Messer, & J.S. Huston. Human single-chain Fv intrabodies counteract in situ huntingtin aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci USA* 98, 4764-4769 (2001)

50. Yamada, O., M. Yu, Y.K. Yee, G. Kraus, D. Looney, & F. Wong-Staal. Intracellular immunization of human T cells with a hairpin ribozyme against human immunodeficiency virus type 1. *Gene Ther* 1, 38-45 (1994)

51. Tavladoraki, P., E. Benvenuto, S. Trinca, D. De Martines, A. Cattaneo, & P. Galeffi. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* 366, 469-472 (1993)

52. Schouten, A., J. Roosien, J. Bakker, & A. Schots. Formation of disulfide bridges by a single-chain Fv antibody in the reducing ectopic environment of the plant cytosol. *J Biol Chem* 277, 19339-19345 (2002)

53. Proba, K., A. Worn, A. Honegger, et al. Antibody scFv fragments without disulfidebonds made by molecular evolution. *J Mol Biol* 275, 245–253 (1998)

54. Visintin, M., G. Settanni, A. Maritan, S. Graziosi, J.D. Marks, & A. Cattaneo. The intracellular antibody capture technology (IACT): towards a consensus sequence for intracellular antibodies. *J Mol Biol* 317, 73-83 (2002)

55. Worn, A., & A. Pluckthun. Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 305, 989–1016 (2001)

Abbreviations: AMCV: artichoke mottled crinkle virus; AS-ODN:antisense oligonucleotides; CDRs: complementarity determining regions; CXCR4: chemokine (C-X-C motif) receptor 4; CCR-5: Chemokine (C-C motif) receptor 5; EGFR: epidermal growth factor receptor; HIV: human immunodeficiency virus; siRNA: small interfering RNA**;** MMP: Matrix Metalloproteinase; NLS: nuclear localisation signal; scFv: single chain variable fragments; RT-PCR: reverse transcription polymerase chain reaction

Key Words: Immunoglobulins, Intrabodies, RNA interference, Cancer, Viral infections, Immune suppression, Gene silencing, Review

Send correspondence to: Dr. Alirio J Melendez, Department of Physiology, Yong Loo Lin School of Medicine, 2 Medical Drive, MD9 #01-05, Singapore 117597, Tel: 65-6516 1697, Fax: 65-6778 8161, E-mail address: phsmraj@nus.edu.sg