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Intrabodies: production and promise

Martin R. Stocks

Antibodies are among the most powerful tools in biological research and are presently the fastest growing category of new drug entities. It has long been a dream to harness their power to probe and modulate activities inside living cells. The binding of an antibody to an intracellular molecule has the potential to block, suppress, alter or even enhance the process mediated by that molecule. In particular, intracellular use of antibody fragments can offer an effective alternative to gene-based knockout technologies, potentially with more control and subtlety of outcome. This article outlines progress in the development of intracellular antibodies or intrabodies and highlights their potential, both as drug-discovery tools and as drug entities in their own right.

Martin R. Stocks
Iclectus Limited
1-3 Burtonhole Lane
London
UK NW7 1AD
e-mail: martin.stocks@iclectus.com

▼ Beginning with the invention of monoclonal antibodies in 1975 [1] and, more recently, the development of various *in vitro* antibody display technologies [2–6], single-specificity antibody has been available in large quantities for biological studies as well as for therapeutic development. Simply by binding to their target, antibodies are capable of numerous effects: blocking molecular interactions, tagging individual target molecules, preventing substrate access to enzymes and preventing (or promoting) enzyme or signalling activity by locking a target molecule into a particular conformation. They can even be created to bind molecular intermediates of chemical reactions, thereby becoming effective catalysts. In principle, whatever can be achieved by antibody binding in the extracellular environment, or in a test tube, should be similarly achievable inside a cell. If active intracellular antibodies were readily available, it would open up new vistas in scientific discovery and drug development. Unfortunately, this essentially simple concept has been very hard to realize in practice. Only recently have technologies developed sufficiently to allow the power of recombinant antibodies to be leveraged to probe and modulate the processes of living cells.

The difficulties of achieving intracellular function are not surprising, when one considers that antibodies have evolved to be secreted from a producing cell, and to function in the extracellular spaces and body fluids. An antibody expressed within the cell cytoplasm has to fold into a highly specific structure without the help of appropriate chaparones, and in a reducing environment that effectively prevents the formation of disulphide bonds, which normally serve to lock the conformation of a correctly folded antibody [7]. However, the potential rewards to be gained from turning the power of antibodies inwards to probe the interior of cells has kept academic and commercial interest high, and in recent years new approaches to antibody screening and construction are finally breaking down the barriers. This review is intended to outline the progress in creating successful intrabodies, and to project forward to visualize the potential uses and value of such entities.

What is an intrabody?

'An antibody functioning inside a cell' would seem at first glance an adequate answer to this question. However, taking a topological and physicochemical view of what constitutes intracellular, we are faced with important distinctions that have to be made when considering the nature of intrabodies and how they are produced. Figure 1 summarizes the nature of the problem. A review of the literature shows that the term intrabody (or intracellular antibody) is used for any antibody that remains within the bounds of the cell membrane. This includes antibody fragments that are secreted into the endoplasmic reticulum (ER), with retention signals (e.g. the KDEL peptide) ensuring that they are retained within the ER–Golgi complex. The purpose of these antibodies is usually to prevent secretion of target proteins, or the maturation of target proteins that would normally be expressed on the cell surface [8–12]. Although this scenario results in an antibody fragment that is, at a gross level, retained within the interior of the cell, topologically the antibody fragment is outside the cell cytoplasm (extracytoplasmic) and it's

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biogenesis will be that of a normal antibody. In particular, the antibody fragment that forms in the lumen of the ER will have access to all the accessory proteins which aid proper folding, and will be capable of forming the intra-chain disulphide bonds that stabilize the structure of the antibody subunits. This is in sharp contrast with an antibody fragment expressed in the cell cytoplasm, which will have no appropriate chaparones available to help it fold correctly and will be incapable of forming disulphide bonds in the strong reducing environment. In practice, very few antibody sequences are capable of folding correctly in the cell cytoplasm, and those that can are the true intrabodies. I would argue that antibodies that are designed to be expressed and function within the ER–Golgi, although valuable reagents in themselves, are not true intrabodies and I would propose that they properly be designated 'retained antibodies' rather than intrabodies.

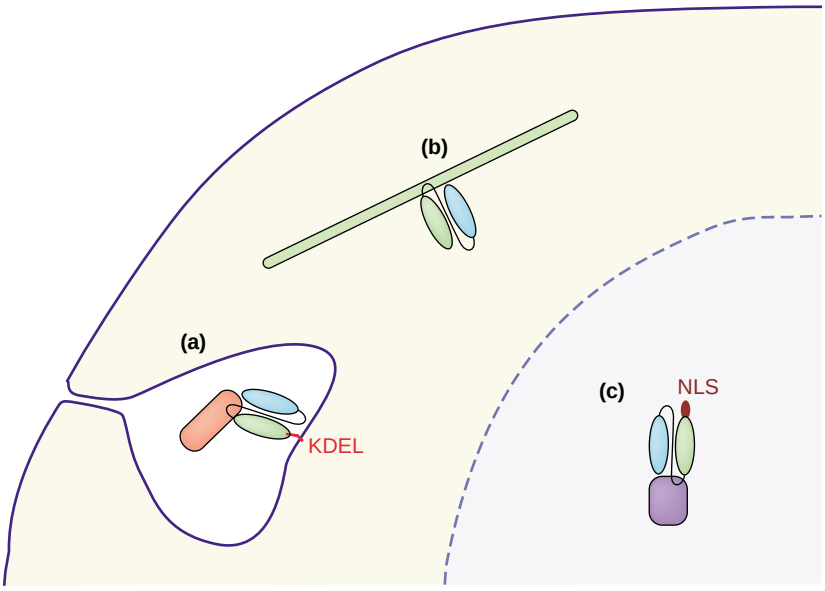

Figure 1. Intracellular antibody fragments can either be extracytoplasmic antibody fragments that are retained in the lumen of the ER or golgi by retention and recycling signals **(a)**; or intracytoplasmic fragments, which have to fold in the cytoplasmic environment **(b)** and can then shuttle to other subcellular locations through the use of cellular localization signals **(c)**. The latter examples are 'true' intrabodies, whereas the extracytoplasmic fragments are referred to as 'retained antibodies' (see text).

A brief history of intrabodies

The first successful attempts at using antibodies intracellularly achieved the goal via the microinjection of intact antibody into cells. This technique, although crude, was remarkably successful in many instances [13]. It is likely that the whole antibody or FAb–FAb' fragments that were injected would in most instances have their intra- and inter-chain disulphide bonds protected, and be sufficiently stable to retain activity on microinjection. However, the laboriousness of the technique and the limited number of cells that could be injected in each experiment rendered this approach of limited use. For intracellular antibodies to be of broad value to biologists and biochemists, expression of antibody from recombinant DNA was required. In 1988 it was reported that an intact, functional antibody could be assembled in the cytoplasm of yeast [14] and, in 1990, the expression of intact antibody in mammalian cells was achieved [15]. This work involved co-expression in COS cells of a λ-light chain gene and a μ-heavy chain after the removal of the signal sequences. This work demonstrated that the two chains were not only expressed but that they associated with one another in the cell cytoplasm with sufficient integrity to be recognizable by an anti-idiotypic antibody. It was further demonstrated that the reconstituted antibody could be redirected to the nucleus by appending a nuclear localization signal to the N-terminus of the light-chain. However, no functional activity was demonstrated for the antibody.

The adoption of the single-chain Fv format as the method of choice for antibody engineering removed one problem with intracellular expression, in that the physical linkage of the heavy and light chain variable regions to form a single polypeptide greatly facilitated the correct association and interaction of the two elements. In the past 15 years there has been a continuous trickle of intrabodies and retained antibodies isolated from phage-display libraries that could be demonstrated to successfully block intermolecular interactions or prevent the proper function or processing of the target antigen (some recent examples are listed in Table 1. and see several recent reviews for a more exhaustive analysis [16–18]). In certain instances the use of localization motifs, sometimes referred to as cellular Zip Codes [19], are essential to the ultimate effectiveness of the intrabody. In the same way that retained antibodies can be held in a specific compartment of the ER–Golgi by retention and recycling signals, intrabodies themselves can be effectively localized within a cell by the use of targeting signals, such as nuclear localization sequences or farnesylation signals [20]. The importance of the subcellular localization of an intrabody is evidenced by work on a group of intrabodies to the oncogenic

Table 1. Recent intrabody publications

Antigen	Intrabody type	Framework structure	Refs
HIV Vif	ICAb	Camel-rabbit hybrid single domain	[36]
Class I MHC	RetAb	scFv	[40]
Huntingtin	ICAb	scFv	[54]
Ron receptor tyrosine kinase	ICAb	scFv	[55]
Cathepsin L	RetAb	scFv	[56]
RDRP	ICAb	scFv	[57]
Hep C NS3	ICAb	svFv	[58]
H-RAS	ICAb	scFv; human V_H single domain	[17,21]
P15 matrix protein of PERV GAG complex	ICAb	Llama single domain	[59]
VEGF receptor type 2	RetAb	scFv	[60]
EGFR cytoplasmic region	ICAb	scFv	[61]

Abbreviations: ICAb: intracellular antibody. RetAb: retained antibody (see text)

$G_{12}V$ mutant form of Harvey RAS (HRAS) that are highly effective inhibitors of HRAS-mediated cellular transformation, but only when directed to the inner face of the plasma membrane by the addition of the same farnesyl transferase substrate sequence as is found in RAS itself. The same intrabodies free in the cytoplasm, although demonstrably capable of binding to HRAS, are considerably less effective at blocking transformation [20].

While scFv intrabodies currently form the bulk of the examples, recent papers have demonstrated that alternate formats can be equally, or even more, effective in some situations. Single antibody domains (e.g. V_H alone, either as naturally occurring sequence or in a 'camelized' modification, or V_L) have been shown to be highly effective intrabodies [21–22]. Alternatively, a multivalent retained antibody, dubbed an 'intradiabody', has been engineered to achieve the simultaneous downregulation of two cell surface receptors, and has been shown to be more effective that the scFv intrabodies that it was derived from [23].

Intrabody structures

It is clear that where successful intrabodies can be generated, they are valuable reagents and could even become therapeutic leads [24]. However, until recently, the ability to isolate a functional intrabody at will from a phage library or by the conversion of a monoclonal sequence has been a major stumbling block, as the majority of antibodies do not survive the transition from isolation *in vitro* to expression in the cell cytoplasm [7]. Thus, there has been considerable effort expended in trying to define what constitutes a successful intrabody and how to reliably isolate or create an intrabody with the desired specificity and binding kinetics.

An intrabody needs to fold properly to tolerate the absence of disulphide bonds, to avoid aggregation and to persist intact within the cell to allow it to express its functional properties. Efforts to generate successful intrabodies have largely fallen into two patterns, the creation of an artificial 'intrabody framework' and the isolation of naturally occurring intrabodies from large libraries. Several groups have studied the relationships between the affinity and structural stability of a specific antibody, and its effectiveness as an intrabody. This has led to the conclusion that the most critical parameter that will allow the intracellular use of a specific antibody is the stability and intrinsic folding capacity of the antibody, with its affinity being of less importance [25]. One can systematically mutate the framework residues of an antibody and define an optimized intrabody framework, which could then be used for the construction of libraries that should contain a high proportion of functional intrabodies [26]. What is not clear from this work is the degree to which CDR sequences and structure impact upon the effectiveness of the framework, and how well new CDRs will be tolerated in the library, although intrabodies with differing specificities have been isolated from a similarly constructed library [27].

The alternative and more empirical approach is to isolate functional intrabodies from a natural repertoire, such as those expressed in large phage libraries, using a combination of *in vitro* and *in vivo* selection strategies [28]. This approach has been highly successful and has yielded many intrabody clones to different antigens. Intriguingly, analysis of the framework sequences isolated by this approach has revealed highly conserved structural elements, and allowed the derivation of a consensus framework, which again has been used to create specific intrabody libraries [29–30]. The diversity of specificities isolated using this technology would suggest that this intrabody consensus framework can sustain a diverse repertoire of CDRs without losing intracellular stability, and this approach has the additional benefit that the intrabody libraries are built on a naturally occurring human framework, which could be a significant advantage if the intrabodies are to be developed as drug leads.

Screening technologies

It is hoped that the creation of antibody fragment libraries using intrabody frameworks, as defined above, will go some way to solve the problems of intrabody isolation. However, whether isolating intrabodies from a naïve repertoire or from a bespoke intrabody library, the one common element in all successful processes to date is the inclusion of an *in vivo* screening step [31,32]. The screening method that currently dominates is a development of the yeast two-hybrid screen, which was originally designed to find interacting pairs in protein networks [33]. This system is ideally suited to intrabody isolation, as the intrabody–antigen interaction is simply a variant of a protein–protein interaction. A target protein (bait) is expressed as a fusion protein with a DNA binding domain, and libraries of antibody fragments (prey) are co-expressed as fusions with a transcriptional activation domain. Binding of antibody to antigen in the yeast nucleus reconstitutes the two parts of a transcription factor and drives the expression of reporter genes – usually auxotrophic markers and/or enzymes which can be assayed for levels of activity. A strength of this approach is that the target antigen does not have to be expressed and purified before selection, as it is expressed from cDNA sequence as part of the screening process.

The main limitation of this technique lies in the efficiency with which yeast can be transformed with DNA. Compared with bacteria, the process of yeast transformation is very inefficient, and the maximum library size that can be screened effectively in a single step by standard protocols is of the order of 10^7 clones, which is <1% of most modern phage display libraries. To use this technology for intrabody isolation from non-intrabody libraries therefore necessitates a multi-step screening process [28], in which a naïve phage-display library is first screened *in vitro*, and then the entire population of binders from a single round of panning is subcloned into the two-hybrid prey vector as a mini-library. Successful binders from the subsequent yeast two-hybrid screen can then be subcloned again for testing in a mammalian two-hybrid screen, which adds an extra layer of stringency (in our hands, typically 20–60% of antibodies that bind antigen in yeast are capable of also binding in mammalian cells). Analysis of panels of intrabody clones isolated by this technique resulted in the definition of the intrabody consensus sequence, described above. Libraries of single-domain (V_H and V_L) intrabodies based on this consensus framework can successfully be screened directly in the yeast two-hybrid system, rendering the *in vitro* phage-display pre-screen unnecessary [21,34]. An alternative approach to overcome the limitations of yeast transformation has involved the screening of naïve libraries using the technique of yeast mating [36].

An alternative approach to intrabody isolation has been espoused recently, which separates the steps of screening for specificity and intracellular function. Gennari *et al*. have attempted to derive intrabodies by screening a very large phage display library *in vitro*, and then pooling the derived clones for assaying intracellularly in mammalian cells, with positive pools then being subdivided and retested to finally arrive at the intrabody clones [32]. Although somewhat more cumbersome than the two-hybrid approaches, and necessitating the production of pure protein antigen for the phage-display panning, this technique has the advantage that it should be capable of isolating all available intrabody clones from a given repertoire, including those that are serendipitously active as opposed to having a generally stable framework.

Deployment: current technologies and future promise

However derived, the greatest issue faced by those who are trying to develop intrabody-based drugs is that of delivery. Current antibody-based drugs are injected and transported around the body to their site of action via the circulation of blood. However, intrabodies need to be within the cell to exert their effects and therefore require an effective vehicle, that will transport them to their site of action and then deliver them to the interior of the target cell population in a functional form. The most obvious solution is expression from a nucleic acid vector as a form of gene therapy, via virus, liposome or naked DNA.

A detailed analysis of the technologies available for the delivery of nucleic acid are beyond the scope of this article, however several recent reviews are available [37–39]. Intrabodies are eminently suitable payloads for almost all available vector technologies, and the small size of each transcriptional unit, coupled with the use of IRES sequences, would allow for the concatenation of multiple intrabody specificities within a single plasmid or virus, even with the smallest of the viral vector genomes, such as AAV The choice of nucleic acid vehicle for intrabody delivery would be determined by the nature of the target and its site of action, rather than by any criteria peculiar to intrabodies. Where the long-term suppression of a target activity was required, an integrating vector such as AAV or lentivirus would be ideal, whereas for the once-only purging of a cell population, an ultra-high expressing RNA virus vector would be ideal. A recent study on human vascular endothelial cells (HUVEC) for seeding vascular grafts has used Adenovirus to deliver a retained intrabody to the HUVEC cells in culture. The intrabody caused a profound downregulation of Class 1 MHC in the cells, even in the presence of cytokines, and prevented allogeneic cell-mediated cytotoxicity [40]. Similarly, adenovirus has been used to deliver an

anti-ErbB2 retained antibody, in a Phase 1 trial for the treatment of ovarian cancer [24].

Considerable effort is being put into the development of non-viral delivery systems, which could also be used for gene-based production of intrabody, technologies that would have many potential benefits, not least the removal of the inherent risks associated with viral DNA delivery, such as insertion-induced oncognesis. Many groups are trying to develop liposome-based systems that would have the stability and efficacy for *in vivo* use in humans, but progress to date has been mixed [reviewed in [38], along with other non-viral transduction technologies. An intellectually attractive, but technically difficult adjunct to the non-viral technologies is the use of antibodies or other ligand-binding molecules to target the encapsulated DNA to particular cell types or physiological locations [41].

Although gene-based delivery of intrabody is a useful laboratory tool, it is likely to be the direct delivery of protein to a target cell population that will bring intrabodies firmly into the fold of mainstream drug development by removing the unfortunate, but all too real, stigma associated with gene therapy. It is technically feasible to deliver protein directly to cells via liposomes or encapsulation by cationic-lipid or peptide, and in one recent report this approach has been used to deliver an antibody to cells in culture [42]. However, the technology with the greatest potential for protein delivery is the use of peptide transduction domains (PTDs).

PTDs are short peptide sequences, or small proteins, which have the capacity to cross the cell membrane and to carry with them other polypeptides. Many such sequences have now been reported, including the herpesvirus VP22 protein [43], and peptides derived from the antennapedia homeodomain protein [44], from the variable regions of anti-DNA autoantibodies [45] and from the tat protein of HIV [46]. The PTDs have a slightly checkered history, with some results being attributed to experimental artifacts [47], and debate about the validity of much of the early work still continues. However, recent experiments have been tightly controlled, and used experimental readouts involving a biological function that could not be a post-fixation artifact. One such experiment involved the delivery of Cre recombinase to cells, the readout being the activation of a reporter gene within the living cells that could only occur by chromosomal rearrangement [48,49]. Similar studies, on proteins containing naturally-occurring PTDs and the use of artificial PTD fusions, are now producing a convincing body of work that demonstrates the utility of PTDs for delivering proteins into cells.

The size of protein that can be delivered by PTD transduction appears to be irrelevant, and certain authors suggest that physicochemical or structural constraints, rather than absolute size, determine whether a particular payload can be delivered effectively [50]. Therefore, it remains to be determined if intrabodies will be suitable payloads for such an approach.

Summary and speculations

The field of intracellular antibodies is one that is currently building momentum following the innovation of stable intrabody frameworks. Their particular strength lies in their capacity to interfere with protein–protein interactions, which underpin most biological processes, from transcription-factor recruitment to viral assembly, and are very difficult to target with small-molecule drugs. Intrabodies have the potential to target molecules that are already well validated as critical to disease processes, but which have been written off as 'undruggable'. The potential uses of intrabodies offer an alluring vision of bespoke therapeutics and powerful tools for high-throughput target discovery and validation.

In this context, comparisons with RNAi, which is currently the 'in-vogue' discovery and validation technology, have to be made. Although no studies to date have directly compared intrabodies with RNAi, there are numerous theoretical distinctions and practical distinctions. RNAi is a cheap, easy to use technology that is very amenable to HTS [51]. However, the early assumptions that all genes would be susceptible to RNAi-based modulation are proving increasingly doubtful, with concerns about off-target effects and other issues such as interferon responses being raised [52,53]. Genes that have very high mRNA-turnover rates, or whose protein products have very low turnover rates (e.g. G-protein-coupled receptors) are very difficult to target effectively with RNAi, whereas intrabodies, which interact with the proteins directly, are unlikely to be affected by target dynamics. Because intrabodies act at the protein level, they will be of value to those wishing to map protein–protein interactions and model drug–target interactions. There is also the intriguing possibility of specifically blocking one function of a multi-epitope target by intrabody binding, while leaving intact the remaining functions or interactions of other domains on that target. Similarly, intrabodies have the theoretical capacity to act as positive regulators of biological processes, for instance by stabilizing active protein conformations or complexes, or tethering a protein in a particular cellular compartment.

The generation of intrabody reagents is now becoming a robust and reliable technology in the hands of a few groups. However, for intrabodies to become therapeutic molecules, the problem of controlled delivery to target cells remains to be solved. Unfortunately gene-based therapy is presently

unpopular with the public, and anathema to the investment community, so there is a need for alternative approaches. This need might be met by new technologies that allow for the direct delivery of the drug entity as protein, via chemical or mechanical vectors.

The next few years are likely to see highly engineered antibodies emerge as one of the most significant classes of biomolecules to enter into the market. It is a question of time before the experimental work on intrabodies achieves the critical mass that will allow us to follow into the mainstream of therapeutics research and drug discovery.

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