Intracellular Antibodies (Intrabodies) and Their Therapeutic Potential

A.S.-Y. Lo, Q. Zhu, and W.A. Marasco(✉)

Abstract Combining exquisite specificity and high antigen-binding affinity, intrabodies have been used as a biotechnological tool to interrupt, modulate, or define the functions of a wide range of target antigens at the posttranslational level. An intrabody is an antibody that has been designed to be expressed intracellularly and can be directed to a specific target antigen present in various subcellular locations including the cytosol, nucleus, endoplasmic reticulum (ER), mitochondria, peroxisomes, plasma membrane and *trans*-Golgi network (TGN) through in frame fusion

W.A. Marasco
Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston MA 02115, USA
e-mail: Wayne_Marasco@dfci.harvard.edu

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with intracellular trafficking/localization peptide sequences. Although intrabodies can be expressed in different forms, the most commonly used format is a single-chain antibody (scFv Ab) created by joining the antigen-binding variable domains of heavy and light chain with an interchain linker (ICL), most often the 15 amino acid linker $(GGGGS)_3$ between the variable heavy (VH) and variable light (VL) chains. Intrabodies have been used in research of cancer, HIV, autoimmune disease, neurodegenerative disease, and transplantation. Clinical application of intrabodies has mainly been hindered by the availability of robust gene delivery system(s) including target cell directed gene delivery. This review will discuss several methods of intrabody selection, different strategies of cellular targeting, and recent successful examples of intrabody applications. Taking advantage of the high specificity and affinity of an antibody for its antigen, and of the virtually unlimited diversity of antigen-binding variable domains available for molecular targeting, intrabody techniques are emerging as promising tools to generate phenotypic knockouts, to manipulate biological processes, and to obtain a more thorough understanding of functional genomics.

1 The Structure of Intrabodies and Antibody Fragments

An intracellular antibody or "intrabody" is an antibody or a fragment of an antibody that is expressed within a designated intracellular compartment, a process which is made possible through the in frame incorporation of intracellular trafficking signals. Intrabodies exert their functions upon exquisitely specific interaction with target antigens. This results in interruption or modification of the biological functions of the target protein. An intrabody can be expressed in any shape or form such as an intact IgG molecule or a Fab fragment (Fig. 1). More frequently, intrabodies are used in genetically engineered antibody fragment format and structures of scFv intrabodies, single domain intrabodies, or bispecific tetravalent intradiabodies are discussed below.

1.1 ScFv and Intracellular Single Variable Domain (IDab)

The most commonly used form of intrabodies is a recombinant scFv Ab in which VH and VL segments are held together by a short, flexible interchain linker (ICL), often the 15 amino acid linker $(GGGGS)_3$ (Fig. 1). A scFv antibody can be in either VH–ICL–VL or VL-ICL-VH configuration and longer ICL (20-mer: GGGGSGGGG SGGGGSSGGGS) has been reported with a VL–ICL–VH orientation (Worn et al. 2000). These formats retain essential regions of antigen-binding specificity of its parental antibody consisting approximately 250 amino acids with a molecular mass $\sim$28 kDa. Because of the extensive sequence and length variation of the complementarity determining region 3 (CDR3), VH domains even have antigen binding activities without their light chain (Ward et al. 1989). An intrabody thus can also be reduced in size to a single functional variable domain. For this designed

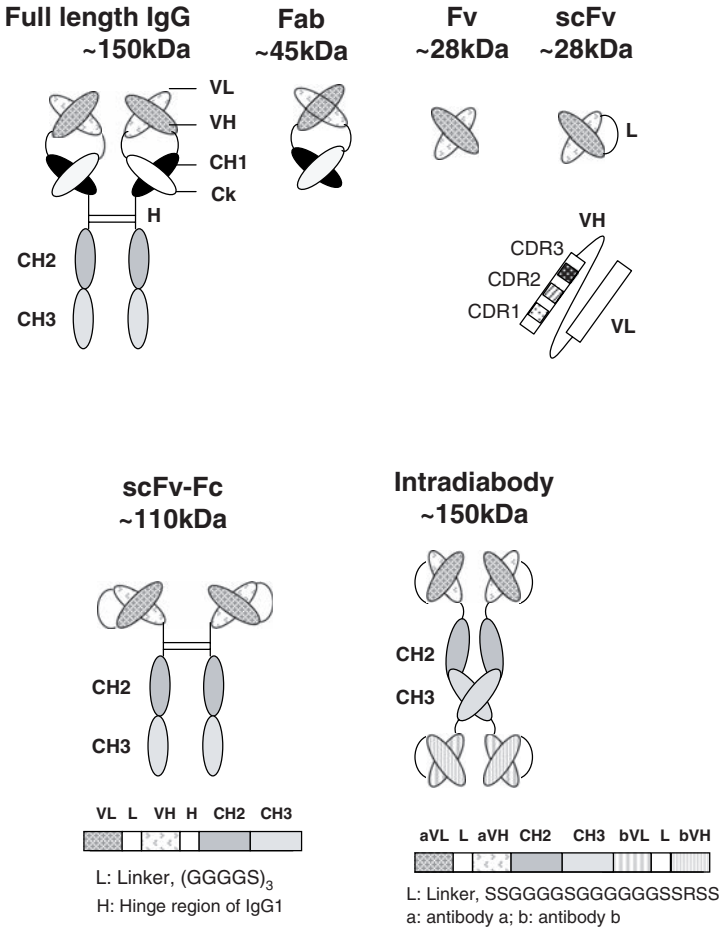

Fig. 1 Structure of antibody and antibody fragments. Intact full length IgG antibody contains heavy chain with variable domain (VH) and three constant domains (CH1, CH2, and CH3), and also the light chain with variable domain (VL) and one constant domain (Cλ or Cκ). One pair of each heavy chain and light chain is held together by interchain disulfide bond within the hinge region (H). Fab fragment is formed without the CH2 and CH3 domain. Single-chain Fv fragment (scFv) is Fv region that contains the variable domains of the heavy and the light chain connected by a linker peptide (L). Complementarity determining regions (CDRs) shape the antigen binding sites and determine the specificity of the antibody. There are three CDRs on each of VH and VL. The CDR1–3 regions of VH domain of scFv are shown. scFv-Fc is an effector antibody, which is composed of scFv and CH2 and CH3 of heavy chain (Fc). Fc domain confers the prolonged half-life of antibody and the Fc-mediated effector functions. The molecular structure is shown as VL–L–VH–H–CH2–CH3. Intradiabody is a bispecific tetravalent antibody with two different scFv antibody fragments (a and b) linked with Fc domain. The molecular structure is shown as aVL–L–aVH–CH2–CH3–bVL–L–bVH

format, a single VH domain, known as an IDab, possesses excellent solubility, stability, and expression within eukaryotic cells. An ideal IDab should exhibit specific antigen recognition and neutralizing activity. IDabs isolated for RAS protein have been shown to inhibit RAS-dependent oncogenic transformation of NIH3T3 cells (Tanaka et al. 2003). In HIV, an important function of viral protein Vif is to suppress the activity of a cytidine deaminase Apobec3G that induces G to A hypermutation in the viral genome and leads to activation of DNA repair mechanisms causing premature degradation of newly synthesized viral DNA. VH single-domain intrabodies against Vif were engineered from rabbit anti-Vif scFv and exhibited a strong neutralization of HIV infectivity and an increase in Apobec3G expression (Goncalves et al. 2002; Aires da Silva et al. 2004). Another format is the Fd fragment (VH-CH1) that has been shown to inhibit glucose-6-phosphate dehydrogenase in the cytoplasm of mammalian cells (Mulligan-Kehoe and Russo 1999). Recently, a VL-Dab and its disulfide bond-free derivative were also successfully utilized as an anti-Huntingtin (htt) intrabody in Huntington's disease (Colby et al. 2004a, 2004b).

1.2 Bispecific Tetravalent Intrabody

Both scFv and IDab are monovalent antibodies. Genetic fusion of scFv or IDab to the IgG Fc region could lead to not only bivalency but also a longer half-life. Multispecific intrabodies have also been engineered. In a recent example, a bispecific tetravalent intradiabody (See Fig. 1, two antivascular endothelial growth factor receptor 2 (VEGF-R2) and two anti-Tie-2 scFv link to the Fc domains of human IgG1) was used to target two endothelial cell receptor-tyrosine kinases: VEGF-R2 and Tie-2. The dual antibody construct simultaneously blocks two independent signaling pathways and affords higher intracellular stability than scFv antibodies. After subtumoral injection of the intradiabody gene carried by a replication incompetent, E1, E3-deleted adenoviral vector and intracellular expression, bispecific intradiabody caused a significant reduction of the growth and angiogenesis of human melanoma M21 in nude mice as compared with a lesser reduction using the monospecific tetravalent intrabody anti-VEGF-R2 over 30 days (Jendreyko et al. 2003; Jendreyko et al. 2005), demonstrating that targeting two different receptors simultaneously yielded an enhanced therapeutic activity.

2 The Molecular Mechanism of Intrabodies

Intrabodies have been used to alter the functions of the target antigens (Marasco, 1995) by modifying related cellular pathways or by redirecting antigen to a new cellular compartment (Zhu et al. 1999; Rajpal and Turi 2001). The unique molecular characteristics of intrabodies allow them to affect protein functions in many ways:

- Sequestration of a target protein from its normal subcellular compartment of action

- Mediating enzyme function through blocking of the active site or modulation of its conformation
- Disrupting biological or signal pathways via interfering normal protein–protein or protein–DNA interactions
- Inducing cell death via activation of the caspase-3-mediated apoptotic pathway
- Selective degradation via the ubiquitin–proteosome pathway

First, intrabodies can be designed to be expressed in different subcellular compartments such as cytoplasm, nucleus, endoplasmic reticulum (ER), Golgi, mitochondria, peroxisomes, plasma membrane, and other locations (Persic et al. 1997). DNA recombinant techniques allow classical intercellular trafficking signals to be genetically fused to the N- or C-termini of antibodies to direct the intrabodies to specific subcellular localizations in order to block or interfere with target antigen function. For instance, ER-retained intrabodies are designed with a signal leader peptide sequence at the N-terminus and a retention peptide, KDEL, at the C-terminus to tether intrabodies within the lumen of the ER. Engineered intrabody can then interact with targeted secretory-pathway proteins, sequestering them within the ER and inhibiting their natural expression. Retention of antibodies in the ER can effectively down regulate its target receptors or signaling molecules (Beerli et al. 1994), such as reduction of cell surface expression of VEGF-R2 and epidermal growth factor receptor (EGFR) (Jannot et al. 1996; Boldicke et al. 2005). Also, ER-targeted intrabody against HIV-1 envelope glycoprotein gp160 blocks HIV-1 envelope processing and virus maturation (Marasco et al. 1993). Similar target protein retention in the *trans*-Golgi has been reported for intrabodies containing a *trans*-Golgi retention signal (Zhou et al. 1998). For expression of cytoplasmic intrabodies, the signal sequences are removed and cytosolic intrabodies are translated on free polysomes. An intrinsically stable amino acid sequence that can fold properly in the absence of disulfide bond formation is required for a cytoplasmic intrabody to function appropriately. Other trafficking signals used to direct antibodies to various specific subcellular locations are listed in Table 1.

Second, intrabodies can modulate enzymatic function by blocking an enzyme active site, by sequestering substrate, or by modulating the conformation of an enzyme catalytic site. It has been shown that cytoplasm-expressed single-domain intrabodies targeting the protein kinase Etk could inhibit its autophosphorylation and ability to phosphorylate its substrate. This resulted in a partial inhibition of cellular transformation in Src-transformed cells (Paz et al. 2005). Third, intrabodies can be used to disrupt biological signaling pathways of target proteins by interfering with normal protein–protein or protein–DNA interactions. For example, a nuclear-targeted intrabody has been used to bind to cyclin-E to inhibit the growth of a breast cancer cell line (Strube and Chen 2002). When genetically fused to caspase-3, intrabodies can be used to promote the death of target cells (e.g. cancer cells) by activating the caspase-3-mediated apoptosis pathway (Tse and Rabbitts 2000). It is also possible for intrabodies to be designed to promote selective degradation of cellular protein targets via the ubiquitin–proteasome pathway by fusion with F-box (Zhou et al. 2000). In addition, intrabodies also have the potential to cause gain-of-function after binding to their target proteins. For example, it has been demonstrated that certain

Table 1 Use of intracellular trafficking signal peptides including leader sequences and retention signal peptides for subcellular compartmental targeting of intrabodies

Subcellular Targeting	The Usage of Leader Sequence and Retention Signal Peptide
Cytoplasm	The immunoglobulin (Ig) leader sequence is removed.
Nucleus	Use of nuclear localization signal (NLS), (T)PPKKK RKV peptide, from the large T antigen of SV40 (Yoneda et al. 1992).
Endoplasmic reticulum (ER)	Use of a signal peptide such as an Ig leader sequence for trafficking to ER and KDEL retention signal for interacting with hERD2 receptor to allow the intrabody effectively tethering within the lumen of ER/Golgi compartment.
Mitochondria	Use of the N-terminal presequence of subunit VIII of human cytochrome C oxidase.
Peroxisomes	Use of the SKL retention signal.
Plasma membrane	Use of the H-Ras or K-Ras CAAX signal to target inner leaflet of the plasma membrane.
Trans-Golgi network (TGN)	scFv fused with 192-bp sequence encoding the transmembrane domain and cytoplasmic tail of TGN38 protein, including the YQRL retention signal of TGN38 protein.

anti-p53 scFv antibodies restored the transactivating activity of mutant p53 in p53 knockout human tumor cells (Caron de Fromentel et al. 1999). As another example, fusion of an anti-p53 scFv intrabody to the DNA-binding domain of bacterial tetracycline repressor resulted in a protein that acted as a transcription activator and inducer of gene expression (Mary et al. 1999). The unique specificity, affinity, and diversity of antibodies make them useful in a wide variety of basic research and clinical applications. More examples of intrabody applications in cancer, HIV, neurodegenerative disease, and transplantation will be discussed in a later section.

3 Practical Considerations for Construction and Selection of an Intrabody

3.1 Intrabody Gene Sources

An intrabody can be constructed by cloning the V-regions of a mouse monoclonal antibody producing hybridoma cell line with a known specificity or can be selected against a specific antigen from antibody libraries of human or animal sources. Immunogenicity of intrabodies that are derived from nonhuman antibody genes is a concern in applications where long-term intrabody expression is desired (e.g., expression of intrabody based HIV resistance genes in $CD4^+$ T cells) but is less of a concern if the intrabody is acting directly in a tumor cell to induce apoptosis/cell killing. In the former example, one likely has to take steps to “humanize”

such antibodies in order to avoid the unwanted immunogenicity as a result of MHC-I presentation of intrabody fragments that may lead to activation of CTL and clearance of the intrabody-expressing cells.

The type and size of recombinant antibody libraries used could also have a direct effect on the success of isolating an intrabody against a specific antigen. "Naïve" or "nonimmune" antibody libraries have been generally built from IgM-V or IgG-V gene pools of B cells of unimmunized individuals, respectively. Rich sources of Ig genes can be from diverse lymphoid organs including bone marrow, peripheral blood, spleen, or tonsils. Early versions of small-size human single-pot libraries with titers of 3×10^7 have been used to isolate antibodies to self-antigens such as thyroglobulin and tumor necrosis factor α (Nissim et al. 1994). However, the antibodies isolated from this library were low in affinity. The larger size library made from over 40 nonimmunized human donors with titer greater than 10^{10} yielded antibodies with higher affinity (Vaughan et al. 1996; Bai et al. 2003). The Marasco laboratory routinely uses the 27 billion member, nonimmune, Mehta I/II libraries that were constructed from peripheral blood B cells from 57 healthy volunteers and have isolated intrabody genes with high affinity and broad epitope diversity (Bai et al. 2003; Gennari et al. 2004). It should be noted that the naïve or nonimmune libraries are biased due to the limited diversity of the Ig repertoire and often unknown exposure history of the B-cell donors. Semisynthetic libraries through CDR randomization, particularly VH CDR3, have been created to address this specific concern (Knappik et al. 2000; Tanha et al. 2001).

In contrast to naïve or nonimmune libraries, the "immune" libraries are constructed from V genes of an immunized animal or human or donors with viral infections, tumors, or autoimmune diseases. Antibodies isolated from these immune sources have been shown to have high affinity and specificity for the antigens under selection since these antibodies have undergone somatic hypermutation leading to their affinity maturation. Using these libraries, antibodies against carcinoembryonic antigen (Chester et al. 1994), major histocompatibility complex/peptide complexes (Yamanaka et al. 1996) and T-cell receptor-Vα (Popov et al. 1996) have been successfully isolated. One limitation of this approach is that this type of library must be newly prepared for each new antigen of interest; however, the approach has been quite useful and has been used for isolating antibodies against self-reactive or toxic antigens (Graus et al. 1997; Wild et al. 2003). For detailed discussion of the types of libraries, their construction and other characteristics, please refer to the reviews cited (Hoogenboom and Chames 2000; Pini and Bracci 2000; Azzazy and Highsmith 2002; Hoogenboom 2005).

3.2 Intrabody Gene Construction

Intrabody constructions, while technically simple to achieve, require a strong understanding of cell biology so that the subcellular compartment that will be used for their translation is properly chosen as this will ultimately effect functional

expression, protein folding, solubility, and stability. During the natural secretory process, the Ig leader sequence directs antibodies to rough ER membranes for synthesis. The nascent polypeptides pass through the ER membrane into the lumen where the leader peptides are cleaved cotranslationally. Upon synthesis, antibody peptides pass through the ER/Golgi while being processed/modified and are normally secreted into the extracellular fluid or remain membrane bound on the B-cell surface as antigen receptors. With the help of chaperones and other factors that are active in the ER, efficient intrachain and interchain disulfide bridge formation and H- and L-chain association occurs.

Cytoplasmic expression of intrabodies is more problematic due to unnatural folding of many antibodies in a reducing environment where disulfide bond formation is inefficient or absent. This can result in low solubility, short protein half-life, and a tendency to aggregate with resulting proteosome degradation (Cattaneo and Biocca 1999). Only intrinsically soluble and stable scFv fragments appear to fold correctly in sufficient amounts to be active as functional intrabodies (Cattaneo and Biocca 1999; Worn and Pluckthun 2001).

At present, no consistent rules have been established that reliably predict which intrabodies will fold and function properly in a cytosolic environment based on primary structure (Marasco et al. 1993); however, preselection allows researchers to identify rare, stable scFvs from natural and engineered scFv-phage libraries. One preselection approach has been use of the yeast two-hybrid system (Visintin et al. 1999; Worn et al. 2000). This method had been further developed for the isolation of leucine zipper binding intrabodies from a library with randomized VH CDR3 sequences located in the stable antibody framework (Auf der Maur et al. 2002). A related approach is to employ stringent selection procedures including denaturation under reducing conditions to isolate antibodies with improved stability from phage display libraries (Brockmann et al. 2005); however, positive antibodies obtained under these harsh elution conditions still need to be functionally tested intracellularly to see if they are effective intrabodies.

Gennari and colleagues (2004) investigated whether specific scFvs that were isolated from a human scFv-phage display library could be directly screened in pools as intrabodies without prior knowledge of their individual identity or purity within pools of antigen-specific scFvs. As the target, they used a synthetic transformation effector site 1 (TES1) polypeptide comprising the membrane-most proximal 34 amino acid residues of the carboxy-terminal cytoplasmic tail of the oncogenic latent membrane protein 1 (LMP1) of Epstein Barr virus. Anti-TES1 scFvs, initially identified by phage ELISA screens, were grouped and then transferred as pools into eukaryotic expression vectors and expressed as cytoplasmic intrabodies. Using this direct phage to intrabody screening (DPIS) strategy they were able to identify intrabodies that were able to selectively block LMP1-induced NFκB activity. This should allow investigators to bypass much of the in vitro scFv characterization that is often not predictive of in vivo intrabody function and provide a more efficient use of large native and synthetic scFv phage libraries already in existence to identify intrabodies that are active in vivo.

Several other strategies have also been developed to address these same concerns. For example, stable and functional cytoplasmic cysteine-free scFv have been generated by using DNA shuffling and phage display (Proba et al. 1998). The VH single domain antibody libraries can also be used to avoid disulfide bond requirement (Tanaka et al. 2003). In addition, a known stable framework was created by point mutations and libraries were generated by grafting synthetic CDRs onto the antibody framework (Ewert et al. 2004). Finally, fusion of intrabodies with *E. coli* maltose binding protein (MBP) were shown to enhance their solubility and stability in bacteria and mammalian cell cytoplasm (Bach et al. 2001; Shaki-Loewenstein et al. 2005). Should bacterial components raise immunogenecity problems in a therapeutic application, the MBP could conceivably be replaced with a mammalian chaperon protein in such a fusion construct.

While scFv have been used in many different kinds of applications as the preferable form of intrabodies, in some studies, it has shown that inclusion of the Cκ light chain constant domain at the carboxy-terminus of a scFv could increase the stability and solubility of an intrabody in cytosol by promoting dimerization (Mhashilkar et al. 1995; Cohen et al. 1998). Reinman et al. (2003) using a bidirectional galactose inducible GAL1–10 promoter to express several formats of antibody fragments against Sem1 (the analog of Sem1P in mammalian cells interacts with tumor suppressor BRCA2) in a yeast system and demonstrated that Fabs gave a higher expression level, function, and stability than scFv or scFv containing the lambda constant domain of L chain (scFvCL). Their studies also suggested that addition of constant region of L or H chain to the scFv increased intracellular levels considerably. This may be explained by the observation that while a conserved hydrophobic patch formed at the variable–constant domain interface (v/c interface) is covered by the constant domains in Fab construct, it becomes exposed in scFv format. The resulting antibody is insoluble, nonfunctional protein produced in the periplasm of *E. coli*. Substitution of a key hydrophobic residue (V84D at VH) of antifluorescein antibody 4-4-20 at the v/c interface significantly improved the in vivo folding of the scFv fragment (Nieba et al. 1997).

It should be noted that the functionality of intrabodies in vivo is often nonrelated or poorly related to their in vitro binding affinity. For example, as compared to a higher affinity scFv intrabody, the intrabody with lower binding affinity possessed greater potency due to its ability to (1) transactivate p53 by inducing a favorable conformational change (Caron de Fromentel et al. 1999) or (2) bind the specific activation domain of Rev for efficient blocking (Wu et al. 1996). A study using two anticaspase-7 scFv demonstrated that an extended half-life and high steady state levels of protein accumulation are critical for functional study of an intrabody (Zhu et al. 1999). It is clear that only in vivo studies can ultimately be used to determine which intrabody is most potent. As a result, intrabodies that bind to different epitopes and with different affinities should be examined early and directly through in vivo functional assays.

3.3 Recombinant Antibody Selection Methods

Many methods can be used to obtain antibody genes for the construction of intrabodies and can be used most effectively if the intrabodies are to be directed to the ER for inhibition of factor secretion or cell surface protein expression. Microbial-based antibody display methods such on phage, yeast, bacterial, and retroviruses as well as nonmicrobial-based display methods such as ribosomal display have been recently reviewed elsewhere by Hoogenboom (2005). During selection, antibodies are enriched by several rounds of panning, consisting of consecutive cycles of incubation with target antigen (either immobilized, soluble, or on surface of cells or paramagnetic liposomes), elution, and amplification.

3.3.1 Lentivirus and Mammalian Cell Display

Mammalian cell surface antibody display is a platform that has gained much attention because its obvious advantage of being able to posttranslationally modify antibody fragments that may contribute to binding affinity and aid proper folding leading to a more diversified antibody repertoire (Ho et al. 2006). With the use of self-inactivating lentiviral vectors, bivalent scFvFc human antibodies were fused in frame with a transmembrane anchoring moiety to allow efficient high-level expression on surface of human cells and lentivirus particle (Taube, Zhu, and Marasco, submitted for publication). Both human cells and virus particles bound antigen in a highly specific manner. FACS sorting in combination with enrichment through magnetic beads allowed isolation of specific scFv expressing cells from a background cell population with 10^6-fold enrichment in a rapid, single round of selection. If necessary, the enriched scFv genes could be immediately recovered by PCR rescue, followed by recloning into a lentiviral display vector, generation of viral particles, and additional rounds of transduction and isolation of antigen-specific scFvs by FACS. Importantly, evidence that the cell surface displayed scFvFc antibodies could indeed undergo posttranslational modification of the variable regions through sulfation of CDR tyrosine residues (Choe et al. 2003; Huang et al. 2004), a property that has been recently shown to markedly broaden the binding affinity and antigen recognition of variable region genes was obtained (Chen, Sui, Zhu, and Marasco, J. Immunol. in press). This antibody display platform should be able to complement existing antibody display technologies by virtue of providing properties unique to lentiviruses and antibody expression in human cells.

3.3.2 Growth Selection Through Protein Fragment Complementation Assay

Protein fragment complementation assay (PCA) has been adapted to screen for antibody binding by reconstituting the activity of dihydrofolate reductase (DHFR) that confers a survival advantage on transformed *E. coli*. In this method, antibodies and antigens are linked with dissected portions of mouse DHFR (mDHFR). The

interaction of antibody and antigen brings the two halves of dissected mDHFR together, thus restoring its enzyme activity and allowing transformed *E. coli* to grow on minimal medium in the presence of antibiotic trimethoprim. Four different target antigens were tested by this system, it was shown that there was about seven orders of magnitude more colonies in antigen pool containing specific antigen as compared with few colonies found in pool with only nonspecific antigen (Mossner et al. 2001). The procedure is relatively simple and fast and only involves transformation of plasmids, functional expression of the fusion proteins, and analysis of the grown bacterial cells. Notably, it gives a very low background of false-positive results. The antigen does not need to be purified and immobilized. However, since the screening is performed in the cytoplasm of *E. coli*, antibodies with inherent stable framework can be isolated under reducing conditions but they would be lack of posttranslational modification.

3.3.3 Growth Selection Through Yeast or Mammalian Two-Hybrid System

Intracellular interaction of antibody with target antigen in yeast could be evaluated by providing conditional cell growth advantage through controlled expression of selected reporter genes (Visintin et al. 1999; Auf der Maur et al. 2002). In such a system, the antigen is usually cloned in frame at C-terminus of the DNA binding domain of Gal 4 (or Lex A) and scFv antibody fused at the N-terminus of a transcription activation domain (AD) of Gal 4 (or VP16). After cotransfection of these plasmids into the yeast cells and upon interaction, the antibody–antigen complex binds to the promoter of reporter genes containing relevant DNA binding sites and activates their transcription. For example, activation of *HIS3* gene controlled by a minimal transcription promoter with Gal 4-binding sites upon antigen–antibody interaction enables the host yeast to grow on plate without histidine and with 3-amino-triazole (3-AT) for selection (Auf der Maur et al. 2002). In addition, Auf der Maur et al. (2001) developed a related procedure in which stable intrabodies could be selected independent of their antigens based on strong correlation between the degree of reporter gene activation and the stability/solubility of the fused antibody. Specifically worth mentioning is a similar system also demonstrated in Hela cells through activation of an integrated luciferase reporter by the strong transcriptional activation domain of the herpes simplex virus type 1 VP16 (VP16-AD) with scFv antibodies fused to its C-terminus and Gal 4 DNA-binding domain at its N-terminus. It should be noted that a library size of 10^7 clones can be screened per assay in these yeast or mammalian systems, which is at disadvantage as compared with $>10^{10}$ clones can be handled with relative ease using phage display.

4 In Vitro or In Vivo Delivery of Intrabody

In vivo and/or clinical applications of intrabody therapy have been limited mainly due to lack of optimal gene transfer vehicles, a common issue in the field of gene

Table 2 In vitro and in vivo expression of intrabodies for intracellular gene targeting

Delivery Method	Intrabody Format and Target (Types of Disease)	Outcome and Reference
Retrovirus	Anti-Tat scFv (HIV)	In retrovirus transduced $CD4^+$-selected, $CD8^+$-depleted, and total PBMC, the anti-Tat scFv expressing cells showed marked inhibition of HIV-1 replication and resistance to HIV-1 infection (Mhashilkar et al. 1999)
Retrovirus	Anti-CCR5 scFv (HIV)	Retrovirus transduced $CCR5^+$ T-cell line, PM1, protected from CCR5-dependent cell fusion and R5 HIV-1 infection (Steinberger et al. 2000)
Retrovirus	Anti-Vif scFv (HIV)	It conferred primary cells highly refractory to challenge with the HIV-1 virus or HIV-1-infected cells and inhibited HIV-1 replication (Goncalves et al. 2002)
Lentivirus	Anti-IL-2Rα scFv Tac (Leukemia)	Using a bicistronic lentivirus vector, the established T-cell line Kit 225 and primary human T cells were shown to have a low or undetectable cell surface expression of IL-2Rα and exhibited a 10-fold reduction of IL-2 responsiveness (Richardson et al. 1998)
Lentivirus pseudotyped with Sindbis envelope	Anti-CCR5 scFv (HIV)	Authors developed lentiviral-derived particles with specificity of gene delivery mediated by pseudotyped Sindbis envelope protein that display scFv recognizing CCR5-expressing cell line and primary lymphocytes in vitro. The nonspecific viral infection was observed by using VSV envelope (Aires da Silva et al. 2005)
Lentivirus	Anti-CXCR4 scFv (HIV)	scFv inhibited infectious entry in primary isolated human brain microvascular endothelial cells (MVECs) and reduced HIV-1 p24 production in postmitotic differentiated human neurons (Mukhtar et al. 2005)
Lentivirus	Anti-CCR5 scFv (HIV)	Lentiviral CCR5 intrabody expression in primary $CD4^+$ T cells were refractory to HIV-1 infection and supported significant growth and enrichment during R5-tropic HIV-1 challenge. Also, thymocytes risen

Table 2 (continued)

Delivery Method	Intrabody Format and Target (Types of Disease)	Outcome and Reference
		from lentivirus transduced $CD34^+$ cells in NOD-SCID-human thymus/liver mice maintained CCR5 intrabody expression and were resistant to HIV-1 challenge (Swan et al. 2006)
Lentivirus	Anti-rat MHC I scFv (Transplantation)	Rat aortic endothelial cells (RAEC) showed a stable expression of the anti-rat MHC I scFv and displayed low MHC I levels. They were protected from killing by allo-specific, cytotoxic T cells (CTL) and by allo-antibody/complement-mediated lysis (Doebis et al. 2006)
Adenovirus	Anti-erbB2 scFv (Ovarian cancer)	ER-directed anti-erbB-2 scFv accomplished specific cytotoxicity in erbB-2-overexpressing tumors. Intraperitoneal injection of adenovirus resulted in a reduction of tumor burdens and prolongation of survival (Deshane et al. 1995b)
Adenovirus	Anti-erbB-2 scFv (Ovarian cancer)	Phase I trial was performed using i.p. administration in erbB-2-overexpressing ovarian cancer. 5/13 patients had stable disease and 8/13 had progressive disease. One patient with nonmeasurable disease normalized her CA125 at the 8-week evaluation and one patient had no sign of disease for 6 months (Alvarez et al. 2000)
Adenovirus	Anti-MHC I scFv (Transplantation)	Intrabody achieved a marked reduction of surface MHC I expression in primary human keratinocytes (Mhashilkar et al. 2002)
Adenovirus	Anti-VEGFR-2 (Angiogenesis)	Adenovirus infected HUVEC showed induction of apoptosis and inhibition of tube formation (Wheeler et al. 2003)
Adenovirus	Anti-MHC-1 scFv (Transplantation)	Adenovirus transduced HUVEC showed a reduced cell lysis in a cytotoxicity assay when compared with that of MHC mismatched control. This effect was not reversed by

Table 2 (continued)

Delivery Method	Intrabody Format and Target (Types of Disease)	Outcome and Reference
		stimulation with inflammatory cytokines (Beyer et al. 2004)
Adenovirus	Anti-αV integrin scFv (Cancer)	Virus transduced melanoma cell line caused detachment of cells from extracellular matrix, induced apoptosis, and prevented subcutaneous tumor implantation in SCID mice (Koistinen et al. 2004)
Adenovirus	Intrabdiabody of anti-VEGF-R2 scFv & anti-Tie-2 scFv (Angiogenesis)	Subtumoral injection of adenovirus gave simultaneous blocking of both VEGF-R2 and Tie-2 and resulted in a significant inhibition of tumor growth and tumor-associated blood vessels (Jendreyko et al. 2003; Jendreyko et al. 2005)
Adenovirus	Anti-Tie-2 scFv pAd-2S03 (Angiogenesis)	scFv inhibited human Kaposi's sarcoma (SLK) and human colon carcinoma (SW1222) xenograft growth in mice and also decreased vessel density markedly in both tumor models (Popkov et al. 2005)
AAV	Anti-Amyloid β scFv (Neurophathology)	AAV injection into corticohippocampal regions of Alzheimer disease (AD) mice gave less amyloid deposits than the mice subjected to PBS injection and showed no eliciting inflammation (Fukuchi et al. 2006)
AAV	Anti-Amyloid β scFv Aβ1-16, 40, 42 (Neuropathology)	Intracranial scFv expression in neonatal amyloid precursor protein mice decreased Aβ deposition by 25–50% and attenuated plaque deposition (Levites et al. 2006)
AAV	Anti-death receptor 5 (DR5) scFv rAAV-S3C (Cancer)	A single i.m. injection of rAAV-S3C in nude mice resulted in a stable secretion of S3C scFv in sera for at least 24 days. scFv suppressed the growth and induced tumor cell apoptosis in established s.c. human lung LTEP-sml and liver Hep3B tumor xenografts (Shi et al. 2006)

therapy that is nevertheless making steady advances into the clinic. Viral vectors including retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV) are currently used to obtain high transduction efficiency and long-term expression of intrabodies (Table 2). While retroviruses produce high transduction level for dividing cells, lentivirus-derived vectors (HIV-1, SIV, EIAV, FIV) can transduce both dividing and nondividing cells including resting T cells, DCs, macrophages, and noncycling hematopoietic stem cells. Lentivirus vectors have been used to express intrabodies against target protein in in vitro studies and result in dramatic reduction of expression and functional activity of target proteins (Table 2). Adenovirus and AAV vectors are also widely used due to their high transduction efficiency, ability to infect a wide variety of cell types, and lack of insertional mutagenesis (Jooss and Chirmule 2003); however, preexisting neutralizing antibodies can significantly reduce the transfer efficiency with these viral vectors. In addition, adenoviral vector induces strong inflammatory responses in vivo. For efficient gene delivery, vectors from different adenovirus serotypes can be used in alternate (Noureddini and Curiel 2005; Bangari and Mittal 2006). Currently, replication incompetent E1, E3-deleted adenoviral vector is the only vector so far used for intrabody gene delivery in a Phase I clinical trial (Alvarez et al. 2000). Compare to adenoviral vectors, although with limited transgene packaging capacity, AAV vectors possess advantages in that they are less immunogenic with a substantially longer lasting gene expression due to their ability to stay extrachoromosomal predominantly as circular concatemers, or in low frequency, to integrate into the host genome (Jooss and Chirmule 2003; Tenenbaum et al. 2003). Originally from rhesus macaques, the new adeno-associated vector rAAV serotype 8 has a very high transduction efficiency by intravenous infusion and avoids the intrinsic immunogenicity against AAV serotypes 1–6 in humans (Gao et al. 2002).

Efficient intravenous targeting to specific cell types is one of the desired gene transfer features for clinical applications. A recent report demonstrated that a Sindbis envelope protein pseudotyped lentiviral vector displaying anti-CCR5 scFv lead to specific targeting to CCR5-expressing cells and primary lymphocytes in vitro (Aires da Silva et al. 2005). P-glycoproteins on metastatic melanoma cells in lung tissue were also successfully targeted by modified lentivirus pseudotyped with a chimeric Sindbis envelope (termed m168) and surface displayed anti-P-glycoprotein antibody through intravenous injection. Unlike other pseudotyped envelope proteins, m168 did not have nonspecific infectivity in the liver and spleen (Morizono et al. 2005). Additionally, by incorporation of an antibody conferring target specificity and a modified influenza hemagglutinin mutant mediating pH-dependent membrane fusion, the lentiviral vector was successfully used to target CD20 in human B cells in vitro and in animals (Yang et al. 2006). Further understanding of envelope tropism and vector trafficking will be important for successful applications of in vivo targeted gene delivery, which, in turn, will accelerate the site-specific expression of therapeutic molecules including intrabodies.

Despite the high transduction efficiency of viral transfer system, viral vectors causes the potential problems including immunogenicity, possibility of insertional mutagenesis, difficulty in large-scale production, and size limitation of exogenous

DNA. Thus nonviral gene delivery systems, although have not been used directly for intrabody delivery, remain as options for the clinical application of intrabody. For example, by linking nucleic acid-binding human protamine to the C-terminus of an anti-erbB2 scFv antibody (Li et al. 2001), exogenous DNA could be selectively delivered into erbB-2 positive cells. Alternatively, an immunoliposome could hold within its lipid bilayer nucleic acids or proteins and has been coupled with antibodies to facilitate targeting and endocytosis to specific cells (Nielsen et al. 2002). Thus immunoliposomes could potentially be utilized for delivery of an intrabody as a gene or a protein. Finally, it is conceivable that an intrabody could also be introduced into cells through protein transduction when fused with short cationic peptide sequences called protein transduction domains (PTD) (Niesner et al. 2002; Lobato and Rabbitts 2003; Joliot and Prochiantz 2004; Heng et al. 2005). This technique eliminates the safety or ethical concerns associated with viral transfer but intrabodies, when delivered as proteins, could be limited in their intracellular level as well as half-life and thus require repeated dosing for an effective treatment.

5 Comparison of Intrabodies and RNAi-Mediated Gene Inactivation

At present, there are two popular technologies for the down-regulation of gene expression – RNA interference (RNAi) and intrabodies. These techniques have shown promising results for biomedical research (Ryther et al. 2005). Researchers choose between these two techniques based on their specific merits and limitations in the context of the desired applications. RNAi is an evolutionarily conserved process of posttranscriptional, sequence-specific gene silencing that uses double-stranded RNA (dsRNA) as an intermediate in the degradation of its homologous mRNA. The silencing effect of dsRNA was first discovered in *Caenorhabditis elegans* and RNAi is now routinely used as a reverse genetics tool in plants (Fire et al. 1998). Gene silencing with RNAi involves two steps. First, long dsRNA are recognized by the ribonuclease III-like enzyme Dicer, which cleaves the dsRNA into small 21–23 bp RNAs. Then these RNAs are associated with helicase and nuclease to form a complex – RNA-induced silencing complex (RISC), which unwinds RNAi and performs sequence-specific degradation of mRNA (Kitabwalla and Ruprecht 2002). It has been reported that RNAi achieves knockdown of gene expression to 10–40% of its normal levels (Coumoul and Deng 2006).

In general, the RNAi strategy follows a simple design with well-defined algorithms and is less technically challenging than intrabody techniques; however, it has nonspecific effects. This nonspecificity takes the form of dsRNA-triggered responses mediated by interferon-associated pathways (Gil and Esteban 2000; Sledz et al. 2003; Sledz and Williams 2004), which do not exist in invertebrates and plants. A gene expression profiling study indicated that $> 1,000$ genes involved in diverse cellular functions are nonspecifically stimulated or repressed in mammalian tissue-culture cells treated with conventional 21-bp RNAi (Sledz et al. 2003; Persengiev

et al. 2004). Another limitation of the RNAi technique is the relatively short half-life of the desired knockdown effects unless stimulatory RNAs are expressed via transfected recombinant DNA (which delays observation of the knockdown effect). In contrast, it is useful to use intrabodies for a nearly instantaneous and durable effect. Intrabodies can block particular binding interactions of target molecules, by changing their structural conformation or by exerting positive functions including catalytic functions, stabilization of protein–protein or protein–DNA interactions, etc. The prominent usages of intrabodies include redirecting target antigen to a particular subcellular location through an appropriate trafficking signal peptide fused with the intrabody (Table 1) and the unique ability to specifically disrupt a specific function of a multifunction protein (Bai et al. 2003). Other molecular mechanisms of intrabodies have been discussed in Sect. 2. Despite the diversity of outcomes elicited through intrabody use, the phage library construction and screening process required to implement intrabody techniques is time consuming and labor intensive. Success in isolation of stable and functional antibody is relatively unpredictable (Sect. 3).

6 Disease Specific Applications of Intrabodies

6.1 Intrabodies in HIV

Intrabodies have important therapeutic potential in microbial pathologies and have been broadly used to interrupt the HIV-1 viral life cycle. ER-directed intrabody F105 targeted to CD4 binding region of HIV envelope protein blocked processing of the envelope precursor gp160 and virus-mediated syncytium formation, leading to low infectivity of progeny HIV particles (Marasco et al. 1993). Later, it was shown that both ER- and TGN-retained anti-HIV-1-gp41 scFv intrabodies inhibited HIV replication and syncytial formation, while only the ER-retained form blocked maturation processing of gp160 into gp120 and gp41 (Zhou et al. 1998). When transduced via a MuLV-based vector, an anti-tat scFv intrabody was more effective than an anti-gp120 scFv in stably inhibiting HIV replication in $CD4^+$ T cells isolated from patients with HIV-1 infection at different disease stage (Poznansky et al. 1998).

Preintegration blockage of virus replication has been demonstrated by intrabodies against the HIV-1 matrix protein (MA, p17) and reverse transcriptase (RT). A Fab intrabody, directed against a carboxy-terminal epitope of MA, p17 from the Clade B HIV-1 genotype, was shown to inhibit HIV-1 infection when it expressed in the cytoplasm of actively dividing $CD4^+$ T cells (Levin et al. 1997). Anti-HIV-1 RT Fab intrabodies expressed in the cytoplasm were shown to block early stage HIV-1 replication in human T-lymphoid cells SupT1. By targeting to a common structural fold of different DNA polymerases, intrabody neutralized RT activity from avian and murine retroviruses, prokaryotic polymerases, and human DNA polymerase α (Gargano et al. 1996).

Postintegration blockage of HIV-1 replication can be achieved by inhibiting critical HIV-1 regulatory protein functions such as Tat-mediated viral transcriptional transactivation or Rev-mediated nuclear export of singly spliced or genomic viral RNA. A cytosolic scFvC_k directed against the N-terminal-activation domain of Tat efficiently inhibited Tat-mediated transactivation of the HIV-1 LTR and resistance to HIV-1 infection in lymphocytes (Mhashilkar et al. 1995). Mhashilkar et al. (1997) demonstrated cooperative down-regulation of HIV LTR-driven gene expression and more durable inhibition of HIV-1 replication in cells with stably expressed anti-tat scFv intrabody when treated with a combination of NF-κB inhibitors pentoxifylline and Gö-6976. Tat binds cooperatively with hCyclin T1, a regulatory partner of cyclin-dependent kinase 9 (cdk9) in the positive transcription elongation factor (P-TEFb) complex, to the transcription response element and is required for HIV transcription elongation. Expression of hCyclin T1-specific scFv intrabodies in SupT1 cells was shown to disrupt the interaction between Tat and hCyclinT1 leading to inhibition of Tat-mediated transactivation and HIV replication. Importantly, the presence of P-TEFb complex indicated that anti-hCyclinT1 intrabody did not disrupt the heterodimerization between hCyclinT1 and Cdk9 (Bai et al. 2003), a prime example to demonstrate intrabody's ability to selectively disrupt a specific function of a multifunction protein while leaving other functions intact. The Rev protein shuttles between the nucleus and cytoplasm of infected cells and is required for the nuclear export of a subset of HIV-mRNAs that encode the structural proteins. A cytosolic directed anti-Rev scFv has been demonstrated to inhibit HIV-1 replication in HeLa-T4 cells (Duan et al. 1994). Wu et al. (1996) showed that a lower binding affinity anti-Rev D8 antibody mapped to activation domain in the C-terminus of Rev had a more potent inhibition of HIV-1 replication in HeLa-T4 cells, human T-cell lines, and PBMC than the higher affinity anti-Rev D10 bound downstream from activation domain in the nonactivation region of the C terminus, demonstrating importance and specificity of an antibody binding site.

Integrase (IN) mediates integration of viral dsDNA into the host genome during early stage of retroviral life cycle. Cytoplasmic or nuclear localized anti-HIV IN scFvs expressed in human T lymphocytes were shown to be resistant to HIV-1 infection by neutralizing IN activity prior to integration (Levy-Mintz et al. 1996). Specific VH single-domain intrabody from immunized rabbit against HIV-1 Vif protein was used to neutralize Vif-mediated enhanced infectivity by reducing late reverse transcripts and proviral integration in nonpermissive cells specifically (Goncalves et al. 2002).

HIV infection requires a coreceptor for entry into permissive cells. CCR5 and CXCR4 are two major coreceptors used by macrophage-tropic and T-cell-tropic HIV strains, respectively. Lentivirus expression of an anti-CXCR4 scFv inhibited infectious entry in primary isolated human brain microvascular endothelial cells (MVECs) and postmitotic differentiated human neurons (Mukhtar et al. 2005). An ER-retained CCR5-specific scFv was shown having a superior effect to RANTES in blocking CCR5 surface expression and cell-to-cell infection in $CCR5^+$ T cell line PM1. Besides, the intrabody ST6 recognizes the conserved region of the first extracellular domain of CCR5 in human and nonhuman primate, thus it could be used

to prevent infection by CCR5-dependent viral infection (Steinberger et al. 2000). In another study, the combinational therapy using anti-CCR5 scFv and hammerhead CCR5 specific ribozyme had an additive effect on both abrogation of the CCR5 cell surface expression and inhibition of higher dose HIV infection (Cordelier et al. 2004). Finally, it is proposed that using a combination of different targeting intrabodies that have a role in different phases of HIV life cycle may achieve additional effects of inhibition. It has been demonstrated that using scFv intrabodies against CXCR4, RT, and IN in combination had a synergistic reduction on HIV-1 infection as compared to the results of using individual scFv (Strayer et al. 2002).

6.2 Intrabodies in Cancer

Intrabodies have been widely used in cancer gene therapy to alter the neoplastic phenotype of cancer cells. This includes knockdown of growth-factor receptors, angiogenesis-related receptors, oncogenic proteins (cell cycle and apoptosis-related), transcription factors, and cancer resistance related proteins.

6.2.1 Growth Factor Receptors and Angiogenesis-Related Receptors

ErbB2 is a member of the type I/epidermal growth factor receptor (EGFR)-related family of receptor tyrosin kinases that include erbB/EGFR, erbB2, erbB3, and erbB4. ErbB2 becomes rapidly phosphorylated and activated following ligand treatment of many cell lines. It is amplified in multiple tumors such as breast and ovarian carcinoma, in which it correlated with a poor prognosis. Expressing ER-retained anti-erbB2 scFv in T47D mammary carcinoma cells resulted in selective reduction of erbB2 cell surface expression and functional inactivation of the receptor by reduction in the phosphorylation of Shc. It also inhibited activation of mitogen-activated protein kinase (MAPK) and p70/p85S6K, and impaired induction of c-*fos* expression in response to natural ligands epidermal growth factor (EGF) and Neu differentiation factor (NDF) (Graus-Porta et al. 1995). No tumor growth was detected and complete tumor eradication was found in mice receiving ER directed anti-erbB-2 scFv 80 days after subcutaneous transplant of human ovarian carcinoma cell line SKOV3 (Deshane et al. 1995a). Treatment with intraperitoneal administration of adenovirus encoding anti-erbB-2 scFv in tumor transplanted mice had shown tumor regression and a prolonged survival as compared with control groups (Deshane et al. 1995b). Phase I clinical trial using this anti-erbB-2 encoding adenovirus (Ad21) for intraperitoneal treatment of ovarian cancer patients demonstrated that 5 out of 13 patients (38%) had stable disease and 8 out of 13 patients (61%) had progressive status of disease. It also showed after the treatment, one patient with nonmeasurable disease remained without clinical evidence of disease for 6 months. Patients generally experienced virus vector related fever without Ad21-specific dose-limiting toxicity (Alvarez and Curiel 1997; Alvarez et al. 2000). Epidermal growth factor

receptor (EGFR) is a member of type I receptor-tyrosine kinase (RTK) family and it is overexpressed in glioblastomas and many epithelial original cancers. ER-targeted scFv against EGFR had been shown to reduce tyrosine phosphorylation of EGFR and cell growth in EGFR transformed NIH3T3 cells (Jannot et al. 1996). Intrabodies were also designed to target a member of the Met RTK family such as Ron (Secco et al. 2004), angiogenesis-related receptors including VEGF-R2 and Tie-2, and another cancer-related folate receptor (Figini et al. 2003).

IL-2Rα (Tac, CD25) plays a key role in T cell-mediated immune response and is constitutively overexpressed in some T- and B-cell leukemias, most notably in adult T-cell leukemia (ATL), which is caused by HTLV-1. An ER-targeted anti-Tac scFv abrogated the cell surface expression completely in PMA-stimulated Jurkat cells (Richardson et al. 1995). IL-2Rα expression was reduced to undetectable levels without affecting cell viability or growth rate (Richardson et al. 1997).

6.2.2 Cell Cycle and Apoptosis-Related Oncogenic Proteins

$p21^{ras}$ is a guanine nucleotide-binding protein, which is involved in the control of cell growth and differentiation. Cytosolic expression of Y259 scFv by removing immunoglobulin leader sequence was shown to perturb $p21^{ras}$ function. Microinjection of mRNA encoding an anti-$p21^{ras}$ scFv intrabody into *Xenopus* oocytes was shown to inhibit insulin-induced meiotic maturation of the cell, a process known to be $p21^{ras}$-dependent (Biocca et al. 1993; Biocca et al. 1994). In other studies, activation of p42 MAPK by *ras* in *Xenopus* oocytes was also strongly inhibited by scFv antibody (Montano and Jimenez 1995). As two scFv Y259 and Y238 mapped to different epitopes of *ras*, Y259 scFv was shown to block *ras*-mediated functions and to elicit an effective tumor regression of HCT116 colon carcinoma cells in nude mice (Cochet et al. 1998b), whereas Y238 scFv was demonstrated to bind to *ras* in oocytes without adverse effect on *ras*-dependent activation pathway (Cochet et al. 1998a).

6.2.3 Signal Transduction

Etk, the endothelial and epithelial tyrosine kinase, is a member of the Tec family of nonreceptor tyrosine kinases, others include Btk, Itk, and Tec. It is involved in several cellular processes including proliferation, differentiation, and motility. Anti-Etk single domain intrabodies was shown to bind specifically to the Etk kinase domain, inhibit its kinase activity, and partially block v-Src-induced cellular transformation in transformed NIH3T3 cells (Paz et al. 2005). The serine-threonine kinase Akt contributes to tumor cell proliferation and survival, and dysregulated function of the PI3K/Akt pathways is commonly found in several human cancers. Intracellular expression of cell-permeable anti-Akt scFv antibodies inhibited p-Ser^{473} Akt and GSK-3α/β phosphorylation, blocked activities of exogenously expressed Akt2 and Akt3, induced apoptosis in three cancer cell lines, and reduced tumor volume

and neovascularization in polyomavirus middle T antigen (PyVmT)-expressing transgenic tumors implanted in mouse dorsal chambers (Shin et al. 2005).

6.2.4 Drug Resistance Proteins

Cancer patients treated with chemotherapy leading to upregulation of cancer multiple drug resistance (MDR) gene. A MDR gene product, P-glycoprotein (P-gp) is an energy dependent drug efflux pump for multiple anticancer agents in human cancers. Anti-MDR1 monoclonal antibody C219 was shown to bind near the ATP binding domain of the cytoplasmic portion of P-gp and inhibit the ATPase activity of P-gp by inhibiting ATP binding. Intracellular expression of anti-MDR1 scFv inhibited the function of P-gp. As a result, the transfected cells exhibited increased Rhodamine123 (Rh123) retention and Adriamycin (ADM) uptake as well as higher sensitivity to ADM (Heike et al. 2001).

6.2.5 Integrins

Integrin heterodimers constituted by αV integrin with one of five different integrin β subunits (β1, β3, β5, β6 and β8) are adhesion receptors for various extracellular matrix proteins including fibronectin, vitronectin, and osteopontin. They are essential for cell anchoring, differentiation, survival, and metastasis. Constructed with KDEL peptide, anti-αV integrin scFv caused a great impairment in cell adhesion to α4β1 ligands and cell spreading on immobilized one of α4β1 ligands – FN40 protein in RD rhabdomyosarcoma cells and Jurkat cells (Yuan et al. 1996). Transfection of anti-αV integrins in osteosarcoma cells resulted in 70–100% reduction in cell surface expression of αVβ3 and αVβ5 leading to reduced cell spreading on fibronectin and vitronectin, induced expression of osteoblast differentiation markers alkaline phosphatase and osteopontin, and suppressed synthesis of gelatinase matrix metalloproteinase-2 (MMP-2) (Koistinen et al. 1999). Finally, expression of anti-αV integrin scFv by adenovirus in melanoma cell lines depleted αV integrins, detached cells from extracellular matrix, and induced apoptosis. Subcutaneous implantation of one of melanoma cell lines transduced with anti-αV adenovirus prevented tumor formation in SCID mice (Koistinen et al. 2004).

6.3 Intrabodies in Transplantation

Major histocompatibility complex (MHC)-restricted antigen presentation is responsible for the rejection of allogeneic cell and tissue transplants. Transplantation of allogeneic MHC class I expressing keratinocytes induces CTL-mediated lysis in response to alloantigen. ER-directed antihuman MHC I scFv intrabody effectively blocked MHC I cell surface expression on monkey and human cell lines with

different HLA-A, B, C haplotypes (Mhashilkar et al. 2002; Busch et al. 2004). Upon transduction by antihuman MHC I scFv encoding adenovirus, susceptibility of primary human keratinocytes to allorecognition by cytotoxic T cells were reduced (Mhashilkar et al. 2002; Busch et al. 2004). Phenotypic knockout of MHC I by intrabody in human umbilical vein endothelial cells (HUVECs) also increased protection of those intrabody-expressing HUVEC from CTL-mediated lysis (Beyer et al. 2004). Furthermore, in xenotransplantation, the carbohydrate structure Galα1,3Gal expressed on pig cells is the major antigen recognized by human xenoreactive natural antibodies (XNA). This activates complement and coagulation cascades and leads to hyperacute rejection of vascularized pig organs in primates. Intracellular expression of anti-α1,3-galactosyltransferase scFv reduced the intracellular accumulation of Galα1,3Gal and its surface expression, thus increased resistance to complement-dependent cytotoxicity mediated by anti-Gal xenoantibodies (Vanhove et al. 1998; Sepp et al. 1999).

6.4 Intrabodies in Neurodegenerative Disease

Abnormal protein aggregation and inclusions in the nuclei of affected neurons is a hallmark of several central nervous system disorders. Huntington's disease is associated with an expanded CAG repeat located within exon 1 of the *IT-15* gene encoding htt. The CAG repeat is translated into a polyglutamine (polyQ) sequence, and abnormal SDS-resistant aggregates with a fibrillar morphology appears when polyQ exceeds more than 37 glutamines (Busch et al. 2003). Intrabodies has been applied to target the proteins related to Huntington's (anti-htt scFv) (Lecerf et al. 2001; Wolfgang et al. 2005) and Parkinson's disease (anti-α-Synuclein scFv) (Zhou et al. 2004) to prevent the misfolding of glutamine-expanded protein and formation of high molecular-weight oligomers, protofibrils and aggregates respectively (Miller and Messer 2005). Lecerf et al. (2001) reported that using nuclear localizing scFv specific to the N-terminal 17 residues adjacent to the polyglutamine of htt successfully reduced length-dependent htt aggregation in cellular and organotypic slice culture models of Huntington's disease. In addition, different domain targeting intrabodies were used to dissect the functional domains of htt. Mouse intrabodies recognized the polyproline region, flanking the polyglutamine on the carboxyl-terminal side, prevented aggregation and apoptosis while intrabodies targeted to expanded polyglutamine stimulated htt aggregation, and induced cell death (Khoshnan et al. 2002). Recently, a single variable light chain (VL)12.3, derived from an scFv against the N-terminal 20 amino acids of htt, was isolated with improved affinity and functional activity by yeast surface-display library (Colby et al. 2004a; Colby et al. 2004b). Another usage of intrabody is targeting to substrate production rather than association with enzyme. ER-targeted intrabody was used to target amyloid β-peptide (Aβ), which is produced as a result of endoproteolysis of the β-amyloid precursor protein by β- and γ-secretases in the brain of Alzheimer's disease patient. (Paganetti et al. 2005). In addition, antiprion intrabodies targeted at ER were shown

to prevent abnormal scrapie isoform PrP^{Sc} accumulation and antagonize scrapie infectivity in mice brain (Cardinale et al. 2005; Vetrugno et al. 2005).

6.5 Intrabodies Targeting Other Viruses

ER-directed anti-hepatitis C virus (HCV) C7-50 scFv bound to the HCV core protein specifically in vitro (Heintges et al. 1999) and this intrabody could be used to study its effect on HCV-replication and virus assembly in hepatocytes. Maedi-visna virus (MMV) is a retrovirus that causes pneumonitis, encephalomyelitis, and arthritis in sheep. Two cytosolic scFv against the transmembrane envelope glycoprotein gp46 of the MMV had been isolated and recognized gp46 peptide in ELISA. These intrabodies have the potential to be used for prevention of the maturation process of the gp150 precursor envelope glycoprotein into gp135 and gp46 in infected cells leading to virus particles with less infectivity (Blazek et al. 2004). Hepatitis B virus X protein (HBx) triggers oncogenesis by transactivating various genes such as *c-fos* and *c-myc*, activating Ras-raf-MAP kinase signaling pathway and promoting cell cycle progression in quiescent mouse fibroblasts. Expression of a scFv targeting to HBx was shown to inhibit HBx-stimulated transactivation in vitro and suppression of tumorigenicity in soft agar and nude mice (Jin et al. 2006).

7 Conclusion and Future Directions

Intrabody technology has been used as a promising tool to achieve a variety of purposes in gene therapy for HIV, cancer, neurodegenerative disease, and transplantation. As gene delivery systems become mature and more sophisticated, intrabody techniques will be used more effectively to achieve phenotypic knockout, neutralization of pathogens, or positive functions. Since intrabody methods operate at the protein level, avenues of research and therapy are possible that would not otherwise have been available through RNA interference (RNAi). Intrabodies also could avoid the RNAi-mediated nonspecific immunologic response that elicits IFN-α signaling pathways. Intrabodies can be expressed inside the cell at defined cellular compartments or to interact with specific structural or functional motifs of a target protein. Moreover, an intrabody method may be the only option in situations where the target molecule has not been cloned or is nonprotein in nature (sugars, DNA, or soluble metabolites). A thorough examination of intrabody stability characteristics or the building of stable phage-display libraries will be of key interest in the field of intrabody engineering. The quality of produced antibody will be dependent on its expression, solubility, and stability. Finally, the single domain intrabody, IDab, which has the most versatile antigen-binding domain, good membrane penetration, and reasonable stability, is becoming an important format for future use.

For future clinical applications, intrabodies could be used therapeutically for ex vivo treatment to suppress MHC molecules in tissue transplants or to down regulate the immunogenicity of adult stem cells for allogenic transplantation. Intrabodies could also be used to alleviate the alloimmune response via $CD8^+$ T cells and infiltrating recipient APC's after transplantation and to purge marrow populations of cancer cells before reinfusion into the patients (Heng et al. 2005). Furthermore, intrabody-mediated gene inactivation will be continuously useful in the investigation of signal transduction of the essential pathologic pathways in diseases or of lineage commitment in cell differentiations. At the time of this writing, high levels of intrabody expression have not been reported to have caused cell death or deteriorated metabolic state. Human or humanized antibodies could be used to circumvent the immune recognition problems. The development of an efficient gene delivery system in conjunction with direct intravenous targeting using viral particles will accelerate the prominent outcomes of intrabody technology.

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