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Targeting antibodies to the cytoplasm

Andrea L. J. Marschall, André Frenzel, Thomas Schirrmann, Manuela Schüngel and Stefan Dübel*

Institute of Biochemistry and Biotechnology; Technische Universität Braunschweig; Braunschweig, Germany

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A growing number of research consortia are now focused on generating antibodies and recombinant antibody fragments that target the human proteome. A particularly valuable application for these binding molecules would be their use inside a living cell, e.g., for imaging or functional intervention. Animal-derived antibodies must be brought into the cell through the membrane, whereas the availability of the antibody genes from phage display systems allows intracellular expression. Here, the various technologies to target intracellular proteins with antibodies are reviewed.

Introduction

Substantial progress has been made in identifying and characterizing the building blocks of cells. It has become apparent, however, that knowing the parts and their interactions is not enough to develop a full understanding of cellular processes, but localization and changes in concentrations of molecules over time need to be considered as well.[1] While standard detection methods that use antibodies to localize proteins require fixation and permeabilization of cells, live cell imaging is needed to study dynamic cellular processes. Initiatives have been started that aim to generate binding molecules that target every human protein and variants, with the goal to resolve the spatiotemporal fate of proteins. Although rabbit sera raised against denatured proteins serve well on fixed tissue sections,[2] their usefulness in functional approaches or analysis in a living cell is very limited. Recombinant antibodies, in particular those selected by phage display, have been proposed as an alternative[3] because they can be raised to correctly folded proteins with significant throughput,[4,5] and they offer additional functional approaches due to the availability of the antibody gene right from the selection. In addition, their biochemical properties can be changed at will, e.g., by fusion to other protein domains, or they can even be immediately expressed inside the living cell to induce knockdown phenotypes.[6,7]

The usefulness of antibodies in visualization or the knockdown of protein functions in the living cell has been proven by microinjection,[8-12] which demonstrated the stability and function of mature antibodies in the living cytoplasm. However, examples published since the first report of this method are scarce, mainly due to the very tedious process, which limits the readout assays to the microscopic observation of individual cells. It is highly desirable to have a broadly applicable method to introduce antibodies into living cells, in particular to make better use of the vast number of antibodies originating from the many ongoing "proteome binder" projects.[3,13] This review evaluates the state of the art of the various approaches designed to deliver an antibody to the inside of living cells (**Fig. 1**).

*Correspondence to: Stefan Dübel; Email: s.duebel@tu-bs.de
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Intrabodies

Antibodies expressed in the cytosol of cells are commonly referred to as "intrabodies," which are distinguished from "retained antibodies" that are expressed intracellularly but not in the cytosol. The introduction of antibodies into the cytosol of living cells would allow tracking at the molecular level and provide the opportunity to interfere with intracellular processes. Because of the great value of these applications, there have been many attempts to express antibodies in the cytosol of cells. In nature, however, antibodies are extracellular proteins that are part of the body's defense system and hence have evolved to be robust molecules capable of surviving in various harsh environments. Their resistance to denaturation is based on a rigid antiparallel β sheet core that is stabilized by disulfide bonds. The formation of these disulfide bonds is essential for the structural integrity and function of the majority of antibodies.[14]

The formation of the correct antibody conformation and disulfide bonds is assisted by endoplasmic reticulum (ER)-resident chaperones such as BiP and the ER-resident protein disulfide isomerase (PDI).[15,16] Consequently, the use of intracellularly expressed antibodies was most successful when antigens in the same secretory pathway were targeted. ER retention is achieved by the addition of an ER retention sequence, e.g., the peptide KDEL, at the carboxy-terminus of the antibody. By inducing retrograde transport from the cis-golgi, the KDEL tag prevents the antibody fragment, and thus its bound antigen, from leaving the ER, which results in subsequent removal of the antigen from the cell surface by normal protein turnover. This knockdown approach is facilitated by the fact that the antibody does not need to inactivate the antigen by binding, but only needs to recognize an accessible epitope on the antigen. The method has been successfully applied to many targets, including human IL2 receptor, ErbB-2 receptor, β-amyloid precursor protein and VCAM1.[6,17-21] Other subcellular compartments could be targeted by adding suitable signal sequences.[19,22] Functional studies of membrane receptors or secreted proteins

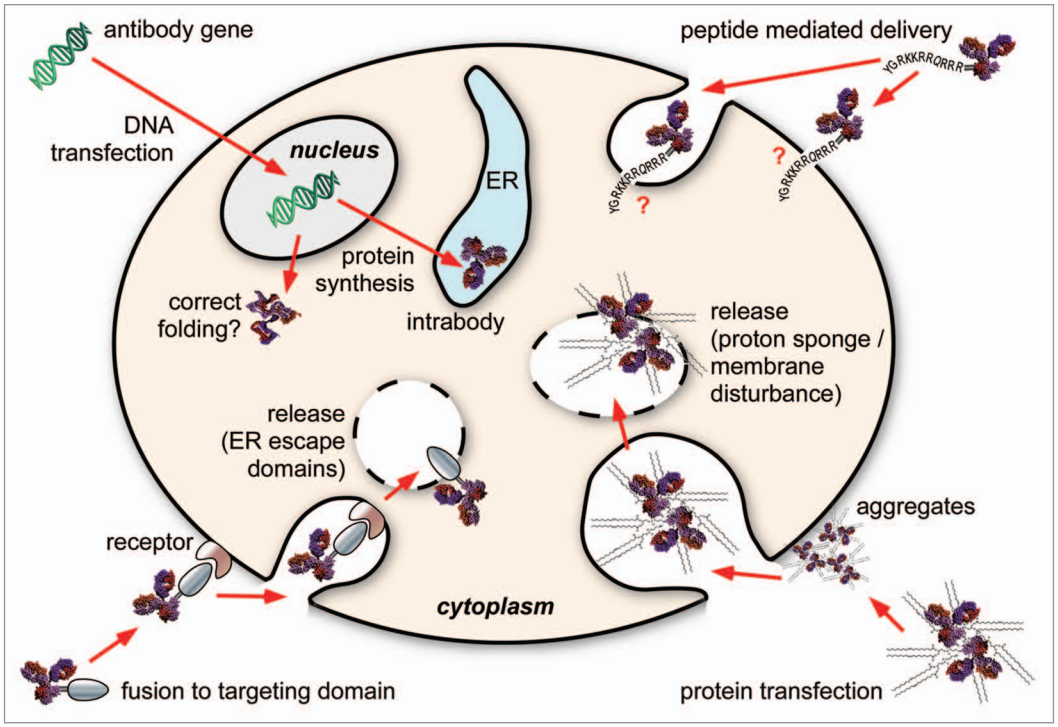

Figure 1. The four major approaches to deliver antibodies into a cell.

can thus be performed using a single standardized subcloning step after selection of a scFv antibody or Fab from a phage display library.

In contrast, the direct expression of antibody fragments in the cytoplasm of the target cell has proven to be much less reliable due to the fact that specialized chaperones needed for antibody folding are lacking and the more reducing milieu impairs the formation of disulfide bonds.[23] The stability of scFvs is sufficient in less than 1% of the cases for use as a high quality intrabody.[24,25] Further, every functional cytoplasmic intrabody needs to be capable of actively inhibiting the target function upon binding, a requirement that makes its selection much more tedious. Despite that, quite a number of studies of cytoplasmic proteins that were successfully targeted and function was affected,[26-29] or were visualized through expression of a scFv-green fluorescent protein (GFP) fusion protein, have been reported.[30]

The problem of incorrect folding and reduced stability of antibodies expressed in the reducing environment of the cytosol has been tackled by different approaches. One approach focuses on determining properties that render antibodies functional in the cytosol and subsequently screening for antibodies with these particular properties. The properties of functional intrabodies for this purpose have been studied. Functional intrabodies may belong to a rare subset of antibodies that do not require intradomain disulfide bonds, as was revealed by functional assays and crystallization of cystein-deleted variants of an anti-RAS intrabody.[31] By comparison of the genes of several different intrabodies, similarities that led to the definition of an intrabody consensus sequence were revealed,[32,33] allowing design of specialized intrabody libraries with diversities in the range of 10^6–10^7.[34]

Selection of intrabodies has been performed using the intracellular antibody capture technology (IACT), which relies on a two-hybrid screen in yeast using the antigen and the scFv as a binding pair.[33] Because the low efficiency with which yeast can be transformed does not allow the screening of complex libraries, a pre-selection of scFvs by panning in *E. coli* must be performed. The clones obtained from the panning are then subjected to a yeast two-hybrid screen and later screened in a mammalian two-hybrid system.[33,35]

Another strategy for selection of intrabodies makes use of the twin-arginine translocation machinery. The twin-arginine pathway (tat pathway) enables the translocation of proteins already folded in the cytoplasm through the bacterial cytoplasmic membrane.[36] The intrabody selection after tat export (ISELATE) technique relies on the fusion of a tat-specific signal peptide (N-terminal) and a β-lactamase (C-terminal) to the scFv. If the scFv is correctly folded and soluble, the fusion protein is exported into the periplasm by the tat-specific signal peptide and confers ampicillin resistance to the cell. In contrast to the two-hybrid based screening, this screening strategy for intrabodies is antigen-independent.[37]

In contrast to the approach that focuses on selection of antibodies with specific "intrabody properties," attempts have been made to convert arbitrary antibodies into intrabodies by fusion to another protein because the approach could be more generally applied.[38] To enhance cytosolic expression, antibodies were fused to a Cκ domain,[39,40] to N utilization substance A (NusA)[41] or to maltose binding protein (MBP).[38] Fusion of a Cκ domain to an anti-p53 scFv led to increased expression levels in the cytoplasm of mammalian cells compared to the anti-p53 scFv alone, which

suggests that the Cκ fusion was less prone to degradation.[39] It remains questionable whether this finding can be generalized because the fusion of another anti-p53 scFv to Cκ did not satisfyingly enhance cytoplasmic expression.[42]

To enhance expression of functional antibodies in the cytosol, antibodies have also been fused to solubility enhancers. NusA and MBP are among the most efficient and validated solubility enhancers for heterologous protein expression in *E. coli*[43,44] and recombinant proteins can be prevented from aggregating if fused to NusA or MBP.[44] However, the folding efficiency of a recombinant protein was found to be more dependent on the properties of the fusion partner than on the solubility enhancer, which puts the universal applicability of the method in doubt.[44] Furthermore, the extent to which solubility enhancers are beneficial is not clear, as the proneness to aggregation is not necessarily an obstacle to the functionality of an intrabody. This is indicated by results obtained with a scFv prone to aggregation, which was reported to be functional by specific co-aggregation with the antigen.[47]

On the other hand, solubility is no guarantee of functionality. Weakened GFP fluorescence was reported to result from expression in *E. coli* as a MBP-fusion protein in spite of improved solubility compared to the unfused GFP.[46] A study reporting the intracellular expression of a scFv-NusA fusion was carried out in a special bacterial strain with an oxidizing cytoplasm, rendering conclusions on the usefulness of this scFv as an intrabody difficult.[41] Inherent properties of individual scFvs are clearly the most important factor influencing whether an antibody functions as an intrabody or not. Another possible reason for the cytosolic stability of an anti-HIV1 TAT scFv, for instance, might be the absence of a high score for "PEST" regions (proline, glutamic acid, serine and threonine rich regions) known to be responsible for rapid proteolysis.[40] Some of the examples given for scFvs that exhibited improved expression upon fusion to tags were already confirmed to be well-functioning intrabodies when expressed without a fusion tag.[40,48,49] Therefore, fusion to a Cκ-domain or to solubility enhancers might lead to enhanced expression or improved performance of an already confirmed functional intrabody, but this method may not be sufficient to convert all scFvs into intrabodies.

Selection of antibodies with intrabody properties, in contrast, comes with the disadvantage of a reduced diversity of the antibody repertoire. The majority of natural antibodies are expected to be non-functional if expressed in the cytosol.[14] In summary, cytosolic expression of antibodies remains a method limited to individual cases, requiring some luck to identify a candidate capable of being folded correctly in the cytoplasm, binding to the target and neutralizing its function. In contrast, intracellular expression of antibodies in the ER has the potential to rapidly develop into a standard method to functionally analyze membrane proteins or the secretome.

Intracellular Antibody Delivery

Since cytosolic expression of antibodies does not reliably result in functional molecules,[14] a generally applicable method that introduces antibodies into the cytosol from outside the cell would be highly desirable, and it would enable use of the growing number of antibodies that target cytoplasmic proteins. The cell membrane, however, represents a non-permissive barrier for antibodies since macromolecules depend on active uptake by the cell. Endocytosed proteins, furthermore, must still be considered "extracellular" because they do not reach the cytosol, but remain in endosomes where they are most likely destined for lysosomal degradation. In order to successfully deliver antibodies into the cytosol, cellular uptake needs to be ensured and endosomal release has to be achieved.

The focus here is on technologies that have been proposed to achieve this goal. The methods are analyzed for their potential to be generally applicable and have been divided into "protein transfection" and protein transduction domain (PTD)-based approaches. The section on protein transfection comprises approaches that employ reagents that are not genetically fused to the protein. Protein transduction approaches based on peptides, which may involve genetic fusion to the antibody fragment, were excluded in this section.

Protein transfection (profection). *Profection under scrutiny.* Over the past 20 years, transfection of DNA has become a robust, standardly used technique to indirectly deliver proteins inside the cell. The direct introduction of proteins by transfection is much more challenging compared to DNA transfection. DNA has comparatively uniform physicochemical properties, but the size, structure and charge distribution of proteins vary over a wide range. Since the association of protein and transfection reagent is necessary for successful delivery to the cell, development of a standard transfection protocol suitable for every protein is difficult due to the highly diverse properties of proteins.[50] Also, unlike DNA, proteins—and antibodies in particular—must retain their tertiary structure upon association with the transfection reagent. An additional difference is that transfected DNA can be amplified by replication of the plasmid, whereas there are no cellular mechanisms for amplification of transfected protein. In the case of protein transfection, the amount of protein actually delivered to the cell has to be sufficient to generate the desired effect.

In spite of the challenges, there are numerous reports of successful intracellular protein delivery. Early studies reported the co-delivery of DNA and proteins such as a transcription factor, the Cre recombinase, nuclear proteins or a polymerase into cells.[51-55] Direct delivery of proteins into cells using cationic lipids and pH-responsive liposomes has been reported as well.[56-61] For example, the inhibition of protein synthesis was achieved by transfection of cells with diphtheria toxin chain A (DTA) incorporated into a pH-responsive liposome. DTA alone, in contrast, did not reach the cytosol, but endosomal escape of the toxin in the absence of diphtheria toxin chain B was dependent on the transfection reagent.[56] Systematic evaluation of a cationic lipid formulation composed of trifluoroacetylated lipopolyamine (TFA-DODAPL) and dioleoyl phosphatidylethanolamine (DOPE) revealed that the mixture could provide delivery of several different proteins into different cell lines.[62] Many protein transfection systems that make use of lipid-based delivery reagents have since been reported, which suggests that the approach is robust (**Table 1**).[63-71]

Table 1. Selected effects after intracellular protein delivery

Protein	Cell line	Observed effect	Reference
anti-N-WASP immunoreagents	Cos-7	Strong reduction of transferrin-endocytosis	Kessels 2002[63]
anti-EGFR phospho-Tyr^{845} IgG	A431 cells	Marked decrease in cell stimulation-dependent phosphorylation of Stat3	Sato 2003[74]
anti-survivin mAb/pAb	HeLa	Microtubule defects, formation of multipolar mitotic spindles, appearance of multinucleated cells	Fortugno 2002, Fortugno 2003[64,75]
c-Fos or c-Jun antibody	quiescent PC12	Completely blocked neurite formation	Gil 2004[65]
Anti-actin IgG Anti Tubulin IgG	BHK HeLa	Depolymerization of cytoskeleton fibers	Dalkara 2004[76]
Dynamitin β-galactosidase as control	Axons of Sensory neurons (primary cells)	Retrograde transport (from axon to cell body) prevented	Heerssen 2004[77]
Monoclonal anti-lamin antibody Avidin-Alexa488	Human U87	Localization at lamin site, formation of micronuclei (change in morphology assumed to be due to partial blockage of lamin function) if high amounts of antibody had been taken up	Didenko 2005[78]
Constitutively active recombinant MEK-1	OK	Increased activation of endogenous ERK, translocation of PKCα to the membrane fraction, increased phosphorylation of the Na^+-K^+- ATPase α_1 subunit, inhibition of Na^+ K^+-ATPase activity	Khundmiri 2005[66]
Cif	HeLa	Enlargement of the cells and formation of actin stress fibers, G_2-arrest	Taieb 2006[67]
Crkl	Splenocytes (primary cells)	Significant ($p < 0.00003$) increase in Stat5b DNA binding ability	Laloraya 2006[79]
Rabbit anti-Cpn0585 or rabbit anti-Chlamydia EB (elementary body) antibodies	HEp-2 cells infected with *C. pneumoniae*	Used for visualization (cells fixed afterwards)	Cortes 2007[80]
Purified human TTase FITC-tagged antibody for determination of transfection efficiency β-galactosidase as a negative control	*TTase*$^{-/-}$ mouse LECs (lens epithelial cells)	Increased TTase activity in *TTase*$^{-/-}$ cells Detoxification of H_2O_2 in TTase loaded TTase$^{-/-}$ cells	Lofgren 2008[69]
Restriction endonucleases	OVCAR SKOV-3	Genome digestion	Geel 2009[81]
NAD^+ glycohydrolase	Neurons (primary cells)	Decrease in cytosolic NAD^+	Alano 2010[71]

ATPase, adenosine triphosphatase; Cif, cycle inhibiting factor; Crkl, v-crk sarcoma virus CT10 oncogene homolog (avian)-like; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; NAD^+, nicotinamide adenine dinucleotide; pAb, polyclonal antibody; PKCα, protein kinase Cα; Stat, signal transducers and activators of transcription; TTase, thioltransferase; WASP, Wiskott-Aldrich syndrome protein.

It is unclear why this approach is not more widely employed since there are numerous promising applications. One of the obstacles previously mentioned is the potential loss of protein activity upon association with the transfection reagent. Tinsley and colleagues reported the use of a polyamine reagent for the transfection of β-galactosidase and GFP into primary coronary venular endothelial cells.[72,73] Contrary to expectations of a loss of protein activity, the transfection reagent had no detrimental effect on β-galactosidase, i.e., the enzyme remained fully active.[72] The observation of cellular effects following protein transfection strongly supports the conclusion that delivered proteins, which include some antibody examples, are functional despite exposure to the transfection reagent.

The observation of some cellular effects also indicates the successful delivery of a sufficient amount of the proteins to the cytosol in these cases. A study describing transfection of an antibody for intracellular staining even described diffuse cytosolic distribution of the transfected antibody that depended on the amount of antibody used for transfection and suggested the presence of unbound excess antibody.[82] It is therefore possible that protein transfection of antibodies may lead to "overstaining" of cells, which belies concerns of insufficient delivery.

Transfection of four different proteins was found to yield optimal results for R-phycoerythrin (R-PE), an antibody, GFP and histone at entirely different ratios of protein to transfection reagent.[50] Although large differences in biochemical properties such as surface charge are seen between individual antibodies, this protein class seems to be well-suited for protein transfections due to their robustness once correctly folded. At the same time, antibodies are highly diverse in their binding specificity, which makes them useful for a vast range of applications. Indeed, many examples of antibody-profections already exist.[50,62-65,74-76,78,80,82-90]

Many of the proposed protein transfection systems are lipid-based, such as TFA-DODAPL:DOPE, dioctadecylglycylspermine (DOGS), combinations of DOPE with CholCSper (a cationic surfactant consisting of cholesterol connected by a cysteine to carboxyspermine) or Saint-2 (N-methyl-4(dioleyl)methyl-pyridinium-chloride), as well as proprietary formulations.[50,62,76,81,91,92] Lipid-based protein transfection was improved by the development of delivery systems with prolonged intracellular release and compatibility with the presence of serum.[91,92] In addition to lipid-based transfection, other strategies for the intracellular delivery of proteins have been successfully employed. A polymer-based transfection that is similar to standard DNA-transfections and employs complexation of proteins with the cationic polymer polyethyleneimine (PEI) has been reported.[78] Use of other carriers such as pH-sensitive carbonate apatite or various nano- and microparticles for intracellular protein delivery,[93-96] as well as a method for protein transfection based on complexation with a peptide (Pep-1, amino acid sequence KET WWE TWW TEW SQP KKK RKV, pI 9.8), have also been described. Similar to cationic lipid-based transfection reagents, Pep-1 contains a hydrophobic part and a cationic part.[97] This approach is a convenient transfection system compared with the substantially more sophisticated procedure required for the production of nano- or microparticles.

Intracellular protein delivery strategies can also be based on protein cationization. This technology involves chemical modification of proteins with diamines or polyamines, which results in a positive net charge of the molecule.[98] The pI of the protein is artificially increased, e.g., by modification with hexamethylenediamine or by covalent linkage of PEI to the protein.[99,100] Cationized proteins can then attach to the negatively charged cell surface by ionic charge interaction and are subsequently taken up by the cell.[100]

Mechanism and parameters affecting profection. The mechanism of protein transfection is believed to be similar to that of DNA-transfection.[62,76,82] The transfection reagent and the biomolecules to be delivered form complexes with a cationic net charge that can interact electrostatically with the negatively charged proteoglycans on the cell surface.[101-103] Attached complexes are then taken up by endocytosis and released from the endosomes either by membrane destabilization caused by lipids or by a "proton sponge effect." [103] The latter was proposed as a mechanism of endosomal release for transfection reagents with high buffering capacity. In the proposed process, protonable N atoms of the transfection reagent take up protons that are responsible for endosome acidification, leading to an accumulation of Cl^--ions, which normally balance the increase of positive charges caused by the influx of protons for acidification, in the endosome. As a consequence, osmotic swelling of the vesicles leads to release of transfected molecules into the cytosol. If polymers are used as transfection reagents, endosome disruption is further supported by expansion of the polymeric material due to repulsive forces between the protonated N atoms.[104]

While the uptake mechanism and endosomal release is thought to be similar in protein- and DNA-transfections, there is a clear difference in the parameters affecting complex formation. In contrast to the importance of the charge ratio of biomolecule to transfection reagent for DNA transfections, the ratio of the surface areas is much more important for protein transfection. The association of proteins with the transfection reagent is not primarily charge-dependent because hydrophobic interactions also seem to be relevant for complex formation of proteins with cationic lipids.[76]

In addition to those that affect complex formation, other parameters can affect transfection efficiency. The transfection process can be subdivided into separate steps, which are (1) association of protein and transfection reagent, (2) contact with cells, (3) uptake into cells and (4) endosomal release. Parameters affecting contact-formation with cells are diffusion or sedimentation, depending on the size of the matter to be transfected. Complexes smaller than about 100 nm are subject to free Brownian motion, whereas larger particles are expected to sediment onto cells.[105,106]

Physical concentration of the transfection reagent at the cell surface is a crucial parameter for transfection-efficiency[107] and transfection efficiency was observed to increase with complex size due to enhanced sedimentation.[108,109] The transfection efficiency can be further enhanced by sedimentation of complexes onto cells employing gravitational, centrifugal or magnetic forces.[107,110,111] After attachment to the cell surface, particle size and cell type influence the uptake.

Depending on their nature and size, complexes enter cells via different routes of endocytosis. Complexes with lipids (lipoplexes) may for instance be taken up by another endocytic pathway than those with polymers (polyplexes).[112] The route of endocytosis is also dependent on the size of complexes. Particles smaller than 200 nm were shown to be internalized via clathrin coated pits, whereas the uptake of larger particles was mediated by caveolae.[113] Endocytic uptake is also dependent on cell type. There can be redundant uptake mechanisms for the same cargo, e.g., for albumin, and the primary route of endocytic uptake can differ depending on cell type.[114]

Release from the transfection reagent after endosomal escape is known to be an important parameter for the efficiency of DNA-transfection.[115] The release of the protein from the transfection reagent has been claimed to be of similar importance.[50,91] While proteins transfected by covalently linked PEI were still functional in the cell and thus required no release from the transfection reagent,[116] post-delivery kinetics appears to be a relevant parameter if proteins form a complex with the transfection reagent. As the prolonged release of proteins by CholCSper:DOPE compared to DOGS showed, different transfection reagents can result in different intracellular release profiles.[91]

Evaluation of profection efficiency. Although there have been attempts to use DNA-transfection reagents for the transfection of proteins, these attempts have resulted in only limited success.[62] Because DNA transfection reagents and procedures must be especially tailored for proteins,[62,76] there is a need for additional studies on profection parameters. Several methods are fundamental to the process of assessing and optimizing the efficiency of protein delivery. In order to determine whether proteins interact with the transfection reagent, gel shift assays similar to those used for the optimization of DNA-transfections have been employed.[78]

As discussed, complex formation and the nature and size of particles are crucial transfection parameters. To reproduce optimal aggregation characteristics, particle sizes and zeta potentials of the protein/transfection reagent complexes have been monitored by electrophoretic light scattering. For quality control, morphology and the supramolecular assembly characteristics have been analyzed by electron microscopy.[91]

Because protein transfection methods are not optimized, attention must be paid to the choice of methods in order to avoid inconclusive results. For example, there are several potential sources of error in procedures used for the detection of recombinant proteins in profected cells. It is necessary to ensure detected proteins are internalized and not only surface-attached. For internalized proteins, the possibility of fixation artifacts that impede correct microscopic localization and the uptake into dead or damaged cells must be excluded, and endosomal release must be confirmed. To reliably determine internalization, exclude fixation artifacts and ensure cell viability, the employment of reporter systems[117] or live cell imaging can be employed. Endosomal escape can also be assessed by monitoring the cellular effects of the delivered proteins. A specific indicator of endosomal escape could be an antibody that binds to a distinct intracellular location that has been validated with other methods. Transfection of a fluorescent anti-nuclear pore complex antibody led to staining of a ring-like structure in cells around the nucleus.[82] In contrast to DNA, some proteins such as some transcription factors and Granzyme B do not require special internalization methods;[118,119] therefore, special attention should be paid to the choice of proteins that are used as model proteins in transfection studies.

Peptides as protein transduction domains—transmabs/transbodies. Protein transduction technology emerged from the discovery that small peptide domains within the homeotic transcription protein Antennapedia and the HIV1 transcriptional activator TAT protein enabled entry into cells.[120] Identification of the minimal sequence motif that confers transduction capability to these proteins led to the discovery of "cell penetrating peptides" (CPPs), which are also called "protein transduction domains" (PTDs). A large number of other PTDs of natural origin or obtained by rational design have since been described. Naturally-derived peptides include the herpes simplex virus structural protein VP22 and antimicrobial or pore-forming peptides.[121] Rational design has yielded peptides such as transportan, which is a chimeric peptide composed of a neuropeptide and a wasp venom peptide toxin.[122]

Despite differences in amino acid sequence, most PTDs share some common physicochemical properties, such as amphipathicity and positive charge.[121] The class of arginine-rich PTDs, which includes HIV1-TAT-PTD, nona-arginine and Antennapedia-PTD, is one of the best-studied and most widely used group of PTDs. Mechanistic aspects mentioned here mainly refer to this class of PTDs. As is the case with most peptidic delivery systems, the exact internalization mechanism is not known. No specific receptor responsible for the uptake of HIV1-TAT-PTD, nona-arginine or Antennapedia-PTD has been identified yet.[123] However, the uptake of HIV1-TAT-PTD was blocked after incubating the target cells with trypsin, which implicates the requirement of a cell surface protein for internalization.[124] Although early results that were potentially affected by methodological artifacts suggested an energy-independent mode of uptake, it is now generally accepted that PTDs are primarily taken up by endocytosis.[121,125] The positive charge of the HIV1-TAT-PTD and other arginine-rich PTDs may help to concentrate the peptide on the cell surface by electrostatic interactions with negatively charged glycoproteins,[124,126-128] similar to the mechanism described for transfection. Heparan sulfate proteoglycans (HSPGs) were proposed to assist in promoting attachment of HIV1-TAT protein and HIV1-TAT-PTD to the cell surface.[124,127,129]

Direct membrane penetration by PTDs is also considered a potential additional mechanism of uptake. Theoretical models for the interaction of HIV1-TAT-PTD with membranes have suggested the formation of transient pores of 3 nm in diameter and a concentration dependence of membrane penetration by peptides.[130] Another simulation predicted the induction of membrane curvature by HIV1-TAT-PTD, potentially leading to internalization of the PTD by vesicle formation rather than pore formation.[131] In experiments with model membranes, HIV1-TAT-PTD entered giant unilamellar vesicles (GUVs) when model membranes contained negative intrinsic curvature lipids such as lipids with phosphatidylethanolamine headgroups.[132,133] Entry into GUVs occurred above a specific concentration threshold of peptides.[133] Experiments carried out with fluorescently labeled arginine-rich PTDs applied to live cells showed a punctuate pattern at 37°C, which indicated endosomal localization of PTDs. In the absence of endocytosis, at 4°C, signals from fluorescently labeled PTDs were not absent but diffusely distributed over the cell. Internalization has consequently been suggested to occur via different routes of endocytosis, potentially including macropinocytosis, clathrin- and caveolin mediated endocytosis, as well as via a mode of entry by direct membrane penetration at high peptide concentrations.[125,133-135] Although direct membrane penetration was proposed as a potential route of entry for PTDs alone, uptake of arginine-rich PTDs linked to macromolecular cargoes appears to occur solely by endocytosis.[127,134,136]

Since routes of entry appear to be similar for most PTDs and delivery efficiency is subject to the same limitations, the potential applications of well-characterized PTDs are worth considering, rather than searching for the "best PTD." As Fischer suggests, mechanistic studies should focus on applications for PTDs rather than "profiling yet another CDV (cell delivery vector) in unconjugated form" and, according to him, "One of the most exciting applications of CDVs would be the delivery of antibodies against intracellular targets." [121] The appealing idea of creating "transbodies" or "transmabs" by linking a PTD to an antibody had already been promoted earlier by others.[137,138] An extensive use of this exciting approach, however, has been missing so far.

Transbodies: why are they so rare? Different antibody formats have been linked to PTDs for intracellular delivery. Single chain variable fragments (scFvs), antigen-binding fragments (Fab) and whole IgGs have been linked to several PTDs, including those derived from HIV1-TAT or Antennapedia protein (**Table 2**).[139-141]

Table 2. Transbodies

Construct	Linkage/Production method	PTD	Cell lines	Reference
Fab	Conjugate	HIV1-TAT(37-62)	A431 breast carcinoma cells	Anderson 1993[139]
$F(ab')_2$	Conjugate	HIV1 TAT(37-72)	Bovine chromaffin cells	Stein 1999[142]
whole IgG	Conjugate	KGEGAAVLLPVLLAAPG ("MTS")	NIH 3T3 cells	Zhao 2001[140]
whole IgG	Conjugate	R_{68}	HeLa cells MCF-7 cells SK-BR-3 cells murine lung enthothelial line NIH 3T3 cells	Chen and Erlanger 2002[143]
scFv	Conjugate	HIV1 TAT (44–57)	HEK293 mice (injection)	Niesner 2002[141]
mAbs, pAbs	Conjugate	KGEGAAVLLPVLLAAPG ("MTS")	Human lymphoma T cells	Zhao 2003[144]
scFv	Genetic fusion, C-terminal	HIV1 TAT(47–57) YGRKKRRQRRR	BMMC, RBL, MCF7	Cohen-Saidon 2003[145]
scFv, radiolabeled	Genetic fusion, 5'-terminal of scFv, produced as inclusion body	HIV1 TAT(47–57) YGRKKRRQRRR	Mice	Nakajima 2004[146]
mAb	Conjugate	HIV1 TAT(47–57) YGRKKRRQRRR	MIN6 β cells	Ohara-Imaizumi 2004[147]
scFv	Fusion	MTS	293T, BT-474 and PyVmT cells	Shin 2005[148]
Fab radiolabeled	Conjugate	HIV1 Tat protein (positions 48–60) Antennapedia (positions 43–58) HIV1 Rev protein (positions 34–50)	HeLa, rats	Kameyama 2006[149]
Fab-Rev radiolabeled	Conjugate	HIV1 REV peptide (positions 34–50) (TRQAR RNRRR RWRER QRGC)	HeLa, rats	Kameyama 2006[150]
IgG	Conjugate	R_{68}	NIH 3T3	Chen 2006[151]
Scfv	Genetic fusion, produced as inclusion body	HIV1 TAT(47-57) YGRKKRRQRRR	Jurkat T cells	Theisen 2006[152]
Fab radiolabeled	Conjugate	TAT (GRKKRRQRRRPPQ-C-amide), REV (TRQARRNRRRRWRERQR-GC-amide) and ANP (RQIKIWFQNRRMKWKK-GC-amide)	HeLa	Kameyama 2007[153]
mAb	Conjugate	GRKKRRQRRRPPQGYGC	MDA-MB-468	Hu 2007[154]
scFv	Antennapedia genetic fusion, C-terminal, immunoaffinity purification	FIRQIKIWFQNRRMKWKK	HCT-116, MRC-5	Avignolo 2008[155]
scFv	Genetic fusion	Penetratin	MDCK cells	Poungpair 2010[156]

Fab, fragment antigen-binding; mAbs, monoclonal antibodies; MTS, membrane transport sequence; pAbs, polyclonal antibodies; PTD, protein transduction domain; scFv, single chain fragment variable.

Antibodies or antibody fragments linked to PTDs have been used to neutralize tetanus toxin in chromaffin cells and influenza A viral activity.[142,156] Further applications included interfering with the cell cycle, such as inhibition of a G_1-S-phase arrest by an anti-Cdk (cyclin dependent kinase) inhibitor,[154] or inhibition of cell cycle progression by an anti-cyclin D1 transbody.[151] Transbodies have also been used to target apoptosis in order to promote, as well as to suppress, apoptosis.[144,145] Apart from interference with intracellular processes, transbodies were used for imaging[140,147] and some studies have focused on the biodistribution of radiolabeled transbodies.[146,149,150] A Fab fragment conjugated to the HIV1-TAT-PTD was reported to exhibit improved retention on tumor cells.[139]

Despite these promising examples, a generally applicable method has yet to be defined, and a possible reason for this may be production problems. Antibodies usually need to be secreted by the producing organism. Although a stable CHO-K1 cell line that secretes TAT-EGFP has been reported,[157] there are also hints at problems with secretion of TAT-fusion proteins. In a study that compared different TAT-fusion proteins (TAT-EGFP, TAT-srIκBα, TAT-RBD), the major fraction of fusion proteins was found in the cell lysate, and only a small amount of the fusion proteins was present in the supernatant.[158] For several variants of HIV1-TAT-PTD with different numbers of cationic amino acids, secretion levels decreased with an increase in cationic charge.[159] Like HIV1-TAT-PTD, most of the known PTDs are cationic in nature. Chemical conjugation was employed for

linking antibody and PTD in most of the examples in spite of the known disadvantages associated with the method, including the inability to control stoichiometry and the precise site of linkage. Importantly, some of the few antibody-PTD fusions reported were not secreted, but produced as inclusion bodies and refolded.[146,152] The production of PTD fusion proteins may further be prone to degradation by the host proteolytic system.[160] Arginine-rich peptides are potential substrates of furin, an endoprotease that resides in the Golgi and cleaves the minimal recognition sequence RXXR. Degradation of the HIV1-TAT-PTD by furin has been observed.[161,162] To solve this problem, a variation of the HIV1-TAT-PTD was proposed in which furin cleavage sites are mutated, but transduction capability is retained.[163]

An additional problem of PTDs is their generally inefficient delivery. High concentrations of PTDs are required for delivery and only small amounts of the PTDs and their cargoes are found in the cytosol. For example, only a vanishingly small fraction of a diphtheria toxin A-PTD fusion was found to reach the cytosol and delivery by the PTD appeared to be at least 10,000-fold less efficient than delivery by diphtheria toxin B.[121,164] The mechanism by which PTDs and their cargoes exit the endosome is still unclear. The HIV1-TAT-PTD is believed to be mainly taken up by macropinocytosis and macropinosomes are believed to be generally "leaky".[165-167] Nevertheless, exit from the endosome remains the rate-limiting step in peptide-mediated delivery, so endosomal entrapment is still a major problem.[121,168]

Perspectives for the application of transbodies. If fusion to a PTD negatively affects secretion of antibodies, then this would be a serious and general disadvantage of the method. The use of universal "adaptors" that can be produced in the cytosol might circumvent the production problems. Protein A fused to HIV1-TAT-PTD (protein A-TAT) is an example of such an adaptor. While it is necessary to produce only one PTD-fusion protein, many different IgG-molecules can potentially enter cells as the cargo of protein A-TAT.[169] Delivery of even larger complexes that consisted of protein A-TAT, an antibody and a third molecule captured by the antibody has been described.[170] Another example of an "adaptor" is the fusion of HIV1-TAT to streptavidin (TAT-SA). Again, the production problem has to be solved for only one fusion protein that may then deliver various biotinylated cargoes into the cell.[171]

In order to overcome endosomal entrapment, several strategies have been proposed, including use of fusogenic lipids, membrane disruptive polymers, lysosomotropic agents, photochemical internalization and membrane disruptive peptides. A combination of PTDs with fusogenic lipids, e.g. DOPE, or membrane disruptive polymers, e.g. PEI, has been used to enhance endosomal release of nucleic acid cargoes.[172] A classic approach to promote endosomal release involves the use of lysosomotropic agents such as chloroquine that prevent acidification of endosomes. At high chloroquine concentrations, endosomes can swell and rupture.[173-175] Other chemicals that act via a different mechanism, including sucrose and dimethyl sulfoxide (DMSO), have also been suggested for enhancing PTD mediated delivery.[176,177]

Photochemical internalization enhances endosomal release by means of "photosensitizers", which are agents that preferentially localize in cell membranes and membranes of endocytic vesicles. Upon irradiation with visible light, reactive oxygen species are formed in the vicinity of the membrane, which leads to rupture of endosomal membranes. A disadvantage of this method is the potential damage to cargo molecules that are close to the photosensitizer,[178] e.g., the enzymatic activity of delivered horse radish peroxidase (HRP) was observed to decrease at higher light doses.[179]

A strategy derived from endosomal escape that occurs in nature involves the use of membrane disruptive peptides. Hemagglutinin (HA), one of the major surface proteins of human influenza virus, consists of two subunits that promote either cell binding (subunit HA1) or endosomal escape (subunit HA2).[180,181] The HA2 subunit, which promotes endosomal release after undergoing a conformational change at acidic pH, has been employed to enhance PTD mediated delivery.[167,181,182] Transduction of a TAT-Cre fusion protein was enhanced by co-incubation with a chimeric peptide consisting of TAT and the HA2 peptide.[167] Increased delivery was also reported for p53 fused to HA2 at the N-terminus and nona-arginine at the C-terminus (HA2-p53-R9) compared with a construct without HA2 (p53-R11). The HA2-p53-R9 construct was employed because of solubility problems with the version containing eleven arginines (HA2-p53-R11).[182] Polyhistidine was used as another membrane disrupting peptide that potentially acts via a proton sponge mechanism. By conjugation of polyhistidine to TAT (TAT-10H), the PTD-mediated delivery of DNA was observed to increase up to 7,000-fold compared with TAT alone.[183]

Avoiding artifacts. Technical artifacts that occurred during the collection of data on the uptake of PTDs or PTD-fusion proteins have been noted. Despite washing, peptides stayed attached to the cell surface, which led to an overestimation of internalized peptides. Surface-bound peptides cannot be distinguished from cytosolic peptides in flow cytometry and these were mistakenly reported as internalized. Misinterpretation of flow cytometry data and fixation artifacts suggested an apparent endocytosis-independent uptake of high efficiency.[121,133] In 2003, Richard and colleagues showed that even mild fixation of cells caused redistribution of membrane parts, which internalized membrane-associated peptides.[184] Consequently, live cell imaging became the preferred method for examination of cells.[168] Reporter systems have also been used to exclude endosomally entrapped PTDs from measurement and to ensure cell viability.[124] To remove cell surface bound PTDs, cells can be either treated with trypsin to digest peptides or subjected to heparin washes for removal of peptides by competitive binding.[185] Fluorescence quenching of cell-surface bound PTDs by application of a membrane impermeant quencher to cells has also been used as a method to exclude external, cell-associated PTDs from measurement.[185,186] Another elegant method to assay internalization based on differential fluorescent labeling of PTD and its cargo was described by Cheung and colleagues.[187] Fluorescent dyes were chosen in such a way as to promote quenching of one dye by the other. Cargo and PTD were linked to each other by a disulfide bond and dequenching by separation of these two components in the reducing environment of the cytosol can be monitored as an indicator of endosomal release.

Table 3. Characteristics of protein transduction domain vs. profection methods

	PTDs	Profection*
Common features	Positive net charge mediates association with cell surface, endocytosis as main route of entrance	
Differences	PTD-fusion proteins are at a molecular scale, covalent linkage of cargo and PTD	Particulate complexes, non-covalent association of cargo and transfection reagent
Efficiency and endosomal release	-	+
Sedimentation techniques for increasing efficiency and reducing toxicity applicable?	-	+
Therapeutic potential	+	-
Cytotoxicity/known side effects	HIV1-TAT-PTD, antennapedia-PTD and nona-arginine inhibit TNF signalling by downregulation of TNF-receptors (TNF receptors are internalized without being activated),[191] penetratin has inhibitory effect on NFκB,[192] dose and exposure-time dependent cytotoxic effects reported for HIV1-TAT-PTD and antennapedia-PTD,[193] poration and intense damage of membranes by transportan.[194]	Cytotoxicity potentially increased compared with DNA-transfections as more lipids are required to complex proteins.[76]

*Not including protein cationization. PTD, protein transduction domain; TNF, tumor necrosis factor.

Profection vs. PTDs—strengths and weaknesses. Profection and PTD-based delivery of antibodies have features in common, but vary in certain aspects; therefore, the two technologies have different strengths and weaknesses (**Table 3**). Whereas PTDs are usually covalently linked to the cargo, transfection reagents attach to their cargo only via non-covalent interactions. The latter poses a serious disadvantage if cargoes are physicochemically diverse, as proteins are. Expression of PTD fusion proteins, in turn, can be subject to production problems, which does not apply to transfection-based approaches. While PTD-linked proteins have sizes at the single molecular scale, profection delivers proteins in the form of particulate complexes. The particulate nature of cargo may be a disadvantage if therapeutic applications are envisaged since these particles are likely to be cleared by phagocytic cells of the reticuloendothelial system[188] or enrich in the liver. Examples of the potential therapeutic applications of PTD-based delivery, in contrast, have already been proposed.[189,190] On the other hand, larger complexes may benefit from sedimentation onto the cells and have the advantage of known increases in transfection efficiency with lower toxicity due to the requirement of decreased amounts of transfection reagent. A major problem of PTD-based delivery is its general inefficiency and endosomal entrapment. In a comparison of delivery efficiency by two profection reagents and two common PTDs, delivery mediated by a lipid based reagent was shown to be 10–20 times more efficient than HIV1-TAT-PTD.[117] As the methods presented might have been studied in varying depth, the effects mentioned might not provide a balanced picture of possible side effects. Because it is likely that no method is entirely free of side effects, studies on potential side effects and reliable controls are the key to harnessing a method in an optimal way.

Fusion to Targeting Proteins

A third strategy to deliver antibodies into the cytosol from the outside is based on the chemical conjugation or genetic fusion with proteins or protein domains that are naturally able to translocate through intracellular membranes, e.g., some ribonucleases or toxins. This principle has been extensively studied for immunotoxins or targeted RNases[195-197] that both require cell binding and uptake into target cells by a ligand or antibody recognizing internalizing surface receptors. After endocytosis, the toxin or RNase moiety must be transferred into the cytosol in order to engage with their cellular substrates and ultimately cause cell death. Bacterial toxins like Pseudomonas exotoxin A (PE) and *Corynebacterium diphtheria* toxin (DT), or the plant toxin ricin from *Ricinus communis* naturally bind and internalize into cells, translocate through intracellular membrane barriers and inhibit protein synthesis by their catalytic domains. PE and DT contain translocation domains that undergo a pH dependent conformational change in the endosome that results in membrane insertion and cytoplasmic release of the catalytic domains.[198,199] Ricin partially unfolds to translocate across the ER membrane via the Sec61p translocon, which is responsible for transfer of misfolded ER proteins back to the cytosol for degradation in proteasomes (ER-associated degradation, ERAD).[200] The translocation is relatively inefficient, but only a few active toxin enzyme molecules delivered to the cytosol are needed to kill the cell.

Certain members of the RNase A superfamily are also able to translocate into the cytosol, but the underlying mechanism is not understood and no distinct translocation domains have been identified so far. It has been suggested that their strong cationic pI helps with uptake into the cell. This hypothesis is supported by studies showing that increased cationization of RNases by chemical modification enhances cytotoxicity and, therefore, cytosolic delivery.[201] In addition, the involvement of distinct cellular retrograde transport mechanisms play an important role. For example, accumulation of cytotoxic RNase in the recycling receptor compartment, rather than in the late endosomes, improves cytosolic release, as do drugs that neutralize the pH of endosomes.[202]

Overall, the translocation of immunotoxins and targeted RNases across intracellular membranes is not very efficient and strategies to deliver other proteins by employing translocation domains of toxins are rare. The efficacy of immunotoxins can

be enhanced by lysosomotropic agents like chloroquine[203] or by saponins that accumulate in endosomal membranes,[204,205] but interference with cellular processes may also lead to artifacts when these methods are used in cell physiological studies. Very recently, the PE translocation domain was successfully used in a tripartite construct. The NEMO-binding peptide was successfully delivered into activated endothelial cells and interfered with the nuclear factor kappaB pathway.[206] Translocation adapter sequences, which are composed of cytosolic and endosomal cleavage sites flanking a viral membrane-penetrating peptide, improved the activity of immunotoxins and targeted RNases more than 100-fold[207,208] and may also be suitable to deliver larger non-toxin protein moieties, including antibody fragments, into the cytosol of cells. Another new approach, receptor-mediated delivery (RMD), utilizes a variant of substance P, a neuropeptide that is rapidly internalized upon interaction with the neurokinin-1 receptor (NK1R), and was able to transfer a chemical conjugated cargo protein into the cytosol.[209] In this study, an antibody was successfully transferred into the cytosol and bound specifically to its antigen, actin. Although this approach is limited to $NK1R^+$ tissues due to overexpression of NK1R in many cancers, it can still be a valuable cell biological tool.

Outlook

The delivery of antibodies into living cells would allow study of protein function and locations in a physiological context and various cells and tissues. However, a general and facile protocol for the delivery of antibodies into a living cell has still to be developed. The most advanced method is the delivery to the ER by DNA transfection with a vector expressing a recombinant antibody fragment that typically is derived from phage display. Functional knockdowns of the antigens were shown to be broadly possible using this method, but restricted to proteins passing the ER. Direct antibody delivery to the cytoplasm is needed to complement this approach and possible solutions to various problems are evolving from a growing body of individual studies, but systematic studies must be done to identify the optimal reagents and establish a robust protocol validated for a set of different antibodies.

Efficient use of transbodies depends on solving production problems and avoiding endosomal entrapment. Among the protein delivery strategies, profection is distinct from the other approaches such as peptide-mediated transduction due to the efficient endosome escape capability generally known for transfection reagents. Several reagents have already been proposed and more than a dozen are commercially available. A systematic comparison of existing protein transfection reagents for antibodies is needed. Recent approaches employing designer translocation sequences, endosome escape domains or peptides targeting cellular receptors capable of transferring cargo proteins through intracellular membrane barriers are promising to improve the cytosolic delivery of antibodies. This would allow better use of the growing number of antibodies from proteome binder projects in the study of protein dynamics and functional knockdowns.

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