

Blocking translocation of cell surface molecules from the ER to the cell surface by intracellular antibodies targeted to the ER

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Received: August 20, 2006; Accepted: November 27, 2006

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Abstract

Intracellular antibodies (intrabodies) constitute a potent tool to neutralize the function of target proteins inside specific cell compartments (cytosol, nucleus, mitochondria and ER). The intrabody technology is an attractive alternative to the generation of gene-targeted knockout animals and complements or replaces knockdown techniques such as anti-sense-RNA, RNAi and RNA aptamers. This article focuses on intrabodies targeted to the ER. Intracellular antibodies expressed and retained inside the ER (ER intrabodies) are shown to be highly efficient in blocking the translocation of secreted and cell surface molecules from the ER to the cell surface. The advantage of ER intrabodies over cytoplasmic intrabodies is that they are correctly folded and easier to select. A particular advantage of the intrabody technology over existing ones is the possibility of inhibiting selectively post-translational modifications of proteins. The main applications of ER intrabodies so far have been (i) inactivation of oncogenic receptors and (ii) functional inhibition of virus envelope proteins and virus-receptor molecules on the surface of host cells. In cancer research, the number of *in vivo* mouse models for evaluation of the therapeutic potential of intrabodies is increasing. In the future, endosomal localized receptors involved in bacterial and viral infections, intracellular oncogenic receptors and enzymes involved in glycosylation of tumour antigens might be new targets for ER intrabodies.

Keywords: cell surface molecules • ER intrabodies • secretory pathway

Introduction

Various approaches are used to study specific protein function: gene-targeted knockout animals and knockdown techniques such as anti-sense RNA, RNAi and RNA aptamers [1–3]. Alternatively,

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Table 1 Intracellular antibodies versus RNA interference and intramers

Intrabodies	RNAi	Intramers
Prerequisite is a specific antibody Time consuming technology	Prerequisite is the sequence of the mRNA or promoter of the target Much less technical challenge	Prerequisite is a single-stranded library of oligonucleotides Much less technical challenge
Very high specificity to the target	Non-specific effects	Very high specificity to the target
Long active half-life	Relatively short active half-life	Relatively short active half-life
Targeting of specific protein domains	Loss of multiple functions of the target	Targeting of specific protein domains
Inhibition of post-translational modifications	Not possible	Maybe possible
No activation of the IFN system known	Sequence and size-dependent activation of the IFN system	Not investigated

intracellular expressed antibodies (intrabodies) can be applied to inhibit protein function [4]. Gene-targeted knockout mice are a powerful experimental system to examine the role of specific genes or protein isoforms [1]. Nevertheless, the generation of knockout mice is very time consuming and in addition the technique is not applicable in human beings compared to the RNAi, RNA aptamers and intrabody technology which might be applicable in human diseases [3, 5, 6].

Intrabodies can be targeted to different subcellular compartments (cytosol, nucleus, ER, mitochondria) by the use of specific signal peptides [4]. Inside the targeted cell compartment, they can exert their function very effectively and specifically. They have the capacity to block or promote protein–protein or protein–DNA interactions [7–9], influence the function of enzymes [9, 10] and inhibit as ER-intrabodies the translocation of cell surface molecules from the ER to the cell surface [4].

This review is focused on the applications of intrabodies targeted to the ER for inhibiting the transport of cell surface molecules to the plasma membrane. In the paper, the intrabody technology is explained and the *in vitro* and *in vivo* applications are reviewed and compared with the gene-silencing technique RNAi and RNA aptamers. Intracellularly applied aptamers are also called ‘intramers’ [11]. The RNAi-mediated gene silencing at the transcriptional and post-transcriptional level is an emerging technology platform, which has become the method of choice for targeted knockdown of gene expression in mammalian cells. RNA interference is mediated by small interfering RNAs (siRNAs), which are intracellularly generated

from long endogenous double-stranded RNA molecules (dsRNAs) through the cleavage activity of a ribonuclease III-type protein [5]. Alternatively, short hairpin RNA (shRNA) are expressed resulting in knockdown of the target message too [5]. Furthermore, the potential and limitations of ER intrabodies as therapeutic reagents are discussed.

The advantages of the intrabody technology are (i) the excellent specificity [12], (ii) very stable expression in mammalian cells (compared to small siRNAs and shRNAs [13] and RNA intramers [14]) and (iii) the possibility to inactivate and study the function of a specific protein domain (Table 1). Specific protein–protein interactions and post-translational modifications of proteins can be targeted that are not possible with gene-targeted knockout animals and knockdown techniques such as RNAi and anti-sense RNA. The RNAi approach is much less technically challenging than the intrabody-mediated knockout of protein function and different vector systems have been developed to allow stable promoter-driven intracellular expression of siRNAs and shRNAs [15, 16]. Furthermore, alleles differing by a single nucleotide polymorphism can be targeted by siRNAs [17, 18], but its major limitation is non-specificity [19, 20]. In addition, transfection of RNAi may result in up-regulation of IFN-stimulated genes [21] depending on the sequence and size of the siRNAs (Table 1).

The advantages of the RNA aptamer/intramer technology is the high specificity, the possibility to select RNAs with a high affinity to the target by an *in vitro* selection (Selex: systematic evolution of ligands by exponential enrichment [3]) and the possibility to

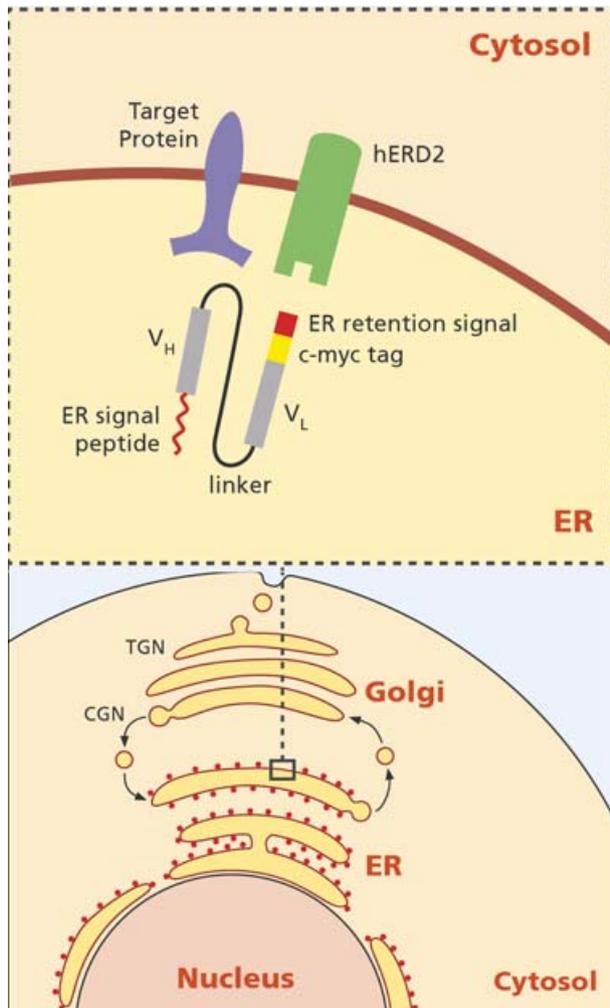


Fig. 1A ScFv intrabody targeted to the ER. Shown is the ER intrabody as scFv fragment. V_H = variable domain of the heavy chain, V_L = variable domain of the light chain. The V_H and V_L domains are fused by a 15 amino acid flexible linker shown as a black line. The red line at the N-terminus of the V_H domain represents the ER signal peptide. The red rectangle at the C-terminus of the V_L domain represents the ER retention sequence and the yellow rectangle the c-myc tag. In addition is shown the target protein (cell surface molecule) and the hERD2 receptor that binds to the ER retention sequence of the scFv fragment. The complex consisting of the scFv fragment and the target protein binds to the hERD2 receptor inside the cis-Golgi and is transported through the Golgi apparatus back to the ER where the scFv-target protein complex is released. (CGN: cis-Golgi Network, TGN: trans-Golgi Network).

target specific protein domains. The main limitation of the intrabody technology is that an antibody against the specific target must exist.

ER intrabodies will give insights into the function of newly detected cell surface molecules and, furthermore, some will have potential application as therapeutic antibodies. A large number of different molecules with specific biological functions are expressed on the cell surface and are involved in cell growth, apoptosis, differentiation, adhesion, bacterial and viral infection and antigen presentation. ER intrabodies are transported to the lumen of the ER and bind to their specific secretory molecule (Fig. 1A). After transport *via* COPII-coated vesicles, the intrabody-target protein complex binds *via* the C-terminal retention sequence (KDEL) inside the cis Golgi network to the human ER receptor hERD2 [22]. Proteins retained inside the ER share a common carboxy-terminal tetrapeptide: KDEL [23]. Initially it was shown that fusion of the sequence SEKDEL from the resident luminal ER protein grp78 to lysozyme led to 100% retention [23]. In addition, fusion of the tetrapeptide KDEL to the secreted protein human proneuropeptide Y (pro-NPY) led to retention, too [24]. For the retention of ER intrabodies, the sequence SEKDEL has been used in almost all studies [4].

The KDEL signal induces oligomerization of hERD2, recruitment of ArfGAP (involved in COPI coat assembly) and formation of COPI-coated budding complexes [25]. The protein complex is then recycled back *via* COPI-coated vesicles to the ER (Fig. 1A). This results in a very efficient down-regulation of the expression of the specific cell surface target molecule on the cell surface. After retrieval to the ER, the proteins dissociate from the receptor and are degraded directly inside the ER [26] or by the cytoplasmic proteasome [27]. Recently, it was shown that the degradation of the intrabody target β -amyloid precursor protein (APP) was blocked by an inhibitor of the cytosolic proteasome [28].

Mouse intrabodies will induce a major histocompatibility (MHC) class I-restricted cytotoxic T-cell immune response in human beings. After degradation of the intrabody molecule, the peptides are complexed with MHC I molecules inside the ER and transported to the cell surface. The MHC class I-peptide complex will then be recognized by cytotoxic T cells that have been primed before by antigen-presenting cells.

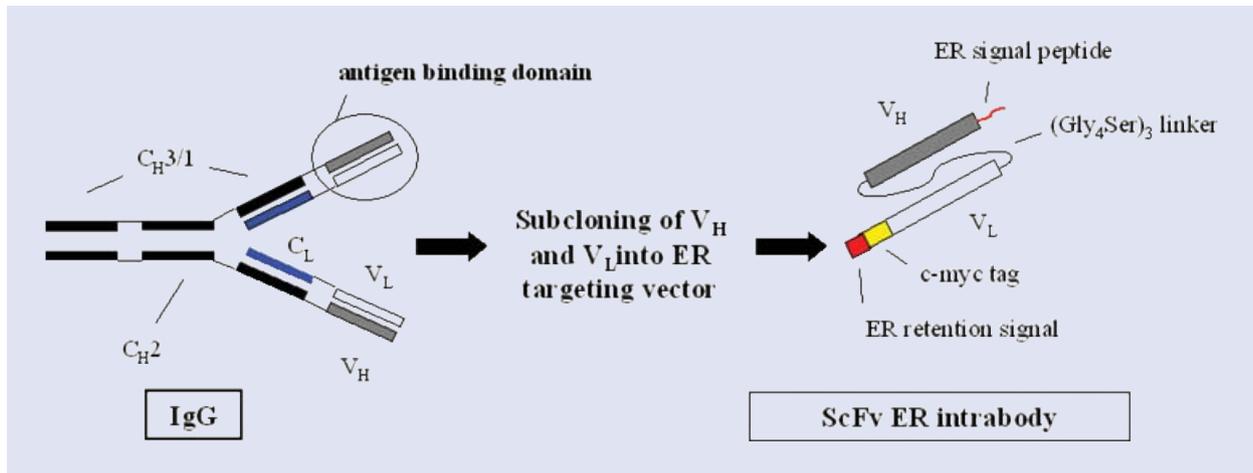


Fig. 1B Construction of an scFv ER intrabody. The scheme shows the construction of an scFv ER intrabody starting from a complete hybridoma antibody. Shown are the heavy chain constant domains $C_{H3/1}$ and C_{H2} , the constant region of the light chain C_L , and V_H and V_L constituting the antigen binding domain. The red square represents the ER retention signal and the yellow rectangle the c-myc tag for detection of the scFv ER intrabody. The 15 amino acid linker assembles the V_H and V_L domains of the scFv fragment.

In the cancer field, ER intrabodies could block the cell surface expression of oncogenically activated proteins [Table 2, 29–46]. They down-regulate viral coat proteins and co-receptors of HIV-1 [Table 2, 47–54] and decrease the immunogenicity of allogeneic cell transplants by inhibiting the transport of MHC I from the ER to the cell surface [Table 2, 55–57]. Intrabodies neutralizing integrins are suitable tools for studying the significance of specific integrins for cell phenotype and differentiation [Table 2, 58–60]. Furthermore, ER intrabodies were successfully targeted to a transmembrane protein involved in the pathogenesis of Alzheimer's disease [28] and to cellular prion protein (PrP^C) [61, 62]. Recently we could show that the function of toll-like receptors (TLRs) could be inhibited by ER intrabodies and have developed an anti-TLR2 intrabody that inhibits very effectively TLR2 signalling (Böldicke *et al.*, manuscript in preparation). Interestingly, some of the cellular proteins targeted by the intrabody approach have been successfully silenced with similar efficiency performed with siRNAs or shRNAs: (ErbB-2, [63], EGFR [64], VEGFR-2 [65], HIV gp120 [66], CCR5 [67], CXCR4 [68], αV integrin [69] and prion-like protein PrP [70]).

Intrabodies expressed in the ER are correctly folded through interaction with molecular chaperones such as BiP and GRP94 and the oxidizing environment of the ER favours intradomain disulfide bond formation [71]. On the contrary, cytoplasmic intrabodies often have folding and stability problems, resulting in low expression levels and limited half-life of antibody domains due

to the reduced environment [72]. To by-pass these problems, the intracellular antibody capture (IAC) technology based on a yeast antibody-antigen two hybrid system was used to select scFv-fragments that tolerate the absence of the intra-chain disulfide bond in the reducing environment of the cytoplasm [73]. Additionally, a stable anti-GCN4 scFv intrabody could be expressed inside the cytoplasm of yeast by grafting the CDR loops to a highly stable single-chain framework [8].

Generation of ER-targeted intrabodies

The main format of an intrabody is the single-chain variable fragment (scFv) of an antibody, which consists of the H- and L-chain variable antibody domain (V_H and V_L) held together by a short, flexible linker sequence (Fig. 1A and B). The source of the intrabody genes are existing hybridomas. To construct the intrabody, gene V_H and V_L can be amplified by an immunoglobulin-specific consensus primer, the scFv fragment assembled by overlapping PCR introducing the linker $(Gly_4Ser)_3$ and cloned into an ER-targeting vector (Fig. 1B). The ER-targeting vector contains a secretory leader, an ER retention signal and a peptide tag (for detection of the antibody, for example c-myc tag) that are both located at the end of the antibody gene (Fig. 1B). The ER retention signal

Table 2 Cellular targets of ER intrabodies**ER Intrabodies inhibiting oncogenic proteins**

Target	Target function	Cellular targets	Performance	References
Human IL-2 receptor	Signal transduction	Jurkat T cells	<i>In vitro</i> studies with intrabody plasmid	[29]
Human IL-2 receptor	Signal transduction	T-cell leukaemia cell line, HTLV-1 transformed cell lines and primary human T cells	<i>In vitro</i> studies with tetracycline responsible intrabody plasmid and HIV-1-based lentivirus vector	[30, 31]
ErbB-2	Signal transduction	NIH/3T3 cells expressing human erbB-2 and mammary carcinoma cells	<i>In vitro</i> studies with murine leukaemia virus vector	[32, 33]
ErbB-2	Signal transduction	Human breast cancer and ovarian carcinoma cell lines	<i>In vitro</i> studies with recombinant adenovirus and intrabody plasmid	[34, 35]
ErbB-2	Signal transduction	Human ovarian carcinoma cells transplanted in SCID mice	<i>In vivo</i> studies with recombinant adenovirus	[36]
ErbB-2	Signal transduction	Human ovarian cancer cells	Phase I study with ovarian cancer patients performed with recombinant adenovirus	[37]
ErbB-2	Signal transduction	Human erbB-2-positive tumour cell lines	<i>In vitro</i> study with intrabody plasmid containing a high affinity anti-erbB-2 scFv fragment	[38]
ErbB-2/androgen receptor	Signal transduction	Human prostate cancer tumour cell line	<i>In vitro</i> studies with murine leukaemia virus vector	[39]
EGFR	Signal transduction	Human tumour cell lines overexpressing EGFR	<i>In vitro</i> studies with murine leukaemia virus vector	[40]
VEGFR-2/KDR	Signal transduction	Porcine aortic endothelial cells overexpressing human VEGFR-2	<i>In vitro</i> studies with intrabody plasmid	[41]
VEGFR-2/KDR	Signal transduction	HUVECs	<i>In vitro</i> studies with recombinant adenovirus	[42]
Tie-2	Signal transduction	Mouse model with Kaposi's sarcoma and human colon carcinoma xenografts	<i>In vivo</i> studies with adenovirus-delivered anti-Tie-2 intrabody	[43]
VEGFR-2/KDR-Tie-2	Signal transduction	Mouse model with human melanoma xenografts	<i>In vivo</i> studies with adenovirus-delivered anti-VEGFR-2-Tie-2 intradiabody	[44, 45]
Human α folate receptor	Signal transduction	Human ovarian carcinoma cell lines	<i>In vitro</i> studies with intrabody plasmid	[46]
Cathepsin L	Endopeptidase	Human melanoma cell lines	<i>In vitro</i> studies with intrabody plasmid	[81]
Metalloproteinase MMP-2 and MMP-9	Degradation of collagen IV	Human lung carcinoma cell line	<i>In vitro</i> studies with intrabody plasmid	[82]

ER intrabodies targeting HIV-1 gp160/gp120 and HIV-1 co-receptors

Target	Target function	Cellular targets	Performance	References
HIV-1 gp120	Viral coat protein	Human lymphocytes and CD4 ⁺ T cells from HIV-positive patients	<i>In vitro</i> studies with recombinant adeno-associated virus and murine leukaemia virus vector	[47, 48]
HIV-1 gp160	Viral coat protein	CD4 ⁺ human T cells (Jurkat)	<i>In vitro</i> studies with intrabody plasmid	[49]
CCR5	HIV-1 co-receptor	CCR5 ⁺ /CD4 ⁺ human lymphocyte cell line	<i>In vitro</i> studies with murine leukaemia virus vector	[50]
CCR5	HIV-1 co-receptor	CD34 ⁺ fetal liver stem cells	<i>In vitro</i> studies with HIV-1-derived self-inactivating lentivirus vector and <i>in vivo</i> studies with NOD/SCID mice transduced with the intrabody expressing CD34 ⁺ fetal liver stem cells	[51]
CCR5	HIV-1 co-receptor	Human monocyte-derived macrophages and microglia cells from human fetal brain tissue	<i>In vitro</i> studies with Tag-deleted SV40-derived vector and hammerhead CCR5 specific ribozyme	[52]
CXCR4	HIV-1 co-receptor	CD4 ⁺ Human T lymphocytic cell line and HeLa-CD4/ βgal-CCR5 cells	<i>In vitro</i> studies with murine leukemia virus and SV40-based virus vector	[53]
CXCR4	HIV-1 co-receptor	Primary human brain microvascular endothelial cells and post-mitotic differentiated human neurons	<i>In vitro</i> studies with HIV-1-based lentivirus vector	[54]

ER intrabodies targeting other viruses as HIV-1

Target	Target function	Cellular targets	Performance	References
Hepatitis C virus core protein	Viral core protein	Human hepatocellular carcinoma cell line and HEK 293 cells	<i>In vitro</i> studies with intrabody plasmid	[83]
Maedi-visna virus gp46 protein	Envelope glycoprotein	Sheep choroid plexus cell line	<i>In vitro</i> studies with the soluble scFv fragment, intracellular ER expression of the antibody is planned	[84]
Root-knot nematode Meloidogyne incognita	Nematode infection	Tobacco leaf protoplasts	<i>In vitro</i> studies with intrabody plasmid	[85]

ER intrabodies abrogating antigen presenting molecules

Target	Target function	Cellular targets	Performance	References
MHC I	Antigen presentation	Primary rat keratinocytes	<i>In vitro</i> studies with intrabody plasmids	[55]
MHC I	Antigen presentation	Human CD4 ⁺ Jurkat T-cell line, human primary keratinocytes and several cell lines of divergent tissue sources	<i>In vitro</i> studies with intrabody plasmid and recombinant adenovirus	[56]
MHC I	Antigen presentation	Human umbilical vein endothelial cells	<i>In vitro</i> studies with recombinant adenovirus	[57]
α 1,3-Galactosyl-transferase	Glycosylation	Pig epithelial kidney cells	<i>In vitro</i> studies with intrabody plasmid	[87]

ER intrabodies targeting integrins

Target	Target function	Cellular target	Performance	References
α V integrin	Mediation of cell-cell and cell-matrix interactions	Saos-2 human osteosarcoma cell line and WM-266-4 melanoma cell line	<i>In vitro</i> studies with intrabody plasmid	[58, 59]
α V integrin	Mediation of cell-cell and cell-matrix interactions	Human metastatic melanoma cell lines and xenograft melanoma SCID mouse model	<i>In vitro</i> and <i>in vivo</i> studies with recombinant adenovirus	[60]

ER Intrabodies targeting proteins involved in Alzheimer's and Prion disease

Target	Target function	Cellular target	Performance	References
Human β -amyloid precursor protein (APP)	Transmembrane protein involved in the pathogenesis of Alzheimer's disease	HEK 293 cells transiently transfected with cDNA of human APP	<i>In vitro</i> studies with intrabody plasmid	[28]
Cellular prion protein	Pathogenesis of Prion diseases	Nerve growth factor-differentiated PC12 cells infected with a suspension of mouse brains from scrapie-affected mice and scrapie mouse model	<i>In vitro</i> and <i>in vivo</i> studies with PC 12 cells stably expressing the intrabody	[61, 62]

(SEKDEL, [23]) retains the antibody inside the lumen of the ER, where it binds the corresponding secretory target molecule and prevents further transport of the antigen to the cell surface (Fig. 1A). In general, expression of the intrabody is performed *via* the CMV promoter. The other intrabody format used is the Fab fragment. Alternatively, scFv fragments can be selected performed with *in vitro* display systems, such as phage, bacterial, yeast cell surface or ribosome display. In addition to the most commonly used intrabody formats, the scFv and Fab fragments, other very

effective antibody formats have now been constructed: a bispecific intradiabody [44, 45] and single-domain intrabodies [74]. The bispecific intradiabody simultaneously silences two independent signalling pathways and shows high intracellular stability compared to scFv intrabodies [44, 45]. Single-domain antibodies are a new generation of small size, stable antibodies that can be isolated from existing phage display libraries.

However, it seems to be not possible to predict if an antibody is fully active as an intrabody.

Nevertheless, we generated two scFv intrabodies recognizing VEGFR-2 from two soluble scFv fragments isolated by phage display [41, 75] and one scFv-intrabody against TLR2 from a complete hybridoma antibody [Böldicke *et al.*, unpublished results]. In all three cases, the intrabodies could inhibit the translocation of the intracellular antigen from the ER to the cell surface to near 100%. Two factors will influence the activity of intrabodies inside the cell: (i) the amount of expressed intrabody and (ii) the stability and structure of the protein. The amount of the expressed intrabody can be controlled by the promoter used. Performed with a stronger promoter as the CMV promoter generally used (for example EF-BOS) could lead to high expression of the intrabody, but could also lead to 'donut-like' aggregates of intrabodies targeted to the cytoplasm or ER compartment [76]. The stability of ER intrabodies (that are correctly folded in contrast to cytosolic intrabodies [72] can be enhanced performed with bivalent formats [44, 45]. In this context, it would be very interesting to compare the stability of an scFv and Fab intrabody fragment. A recent published paper shows that the change of an scFv fragment to a Fab format improves the *in vivo* stability significantly [77].

Blocking translocation of growth factor receptors

Numerous key molecules, proto-oncogenes, hormones, peptides, growth factors and their corresponding receptors expressed by cancer cells, are involved in the proliferation of these cells [78]. Growth factor receptors, often carrying tyrosine kinase activities in their cytoplasmic domains, are overexpressed in many cancers and have been successfully targeted by intrabodies. Cell growth and differentiation is induced via intracellular signalling after binding of the specific growth factor to the extracellular domain of the receptor [79]. Performed with ER-intrabodies, the function of oncogenic receptors and the corresponding tumour growth was inhibited (for example: IL2 receptor [29–31], ErbB-2 [32–39], EGFR [40], VEGFR-2 [41, 42], Tie-2 [43], simultaneously knockout of VEGFR-2/Tie-2 [44, 45] and human α -folate receptor [46]).

Overexpression of ErbB-2 is observed in many tumours including breast and ovary. Intraperitoneal

delivery of adenovirus encoding an anti-erbB-2 intrabody enhances survival and reduces tumour growth in a xenograft model of human ovarian carcinoma in SCID mice [36]. A phase I trial with ovarian cancer patients shows the limitations of an *in vivo* approach mainly due to insufficient adenoviral gene transfer [37]. Furthermore, performed with the ER intrabody approach, the crosstalk between ErbB-2 and the androgen receptor (AR) was experimentally verified. Expression of an ER-targeted anti-erbB2 intrabody in LNCaP prostate cancer tumour cells inhibited androgen-stimulated recruitment of AR to the androgen responsive enhancer of the prostate specific antigen (PSA) gene and histone acetylation of the enhancer, endogenous (PSA) expression and androgen-stimulated growth [39]. It showed that ErbB-2 signalling is required for optimal transcriptional activity of AR in prostate cancer cells.

The human vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) and the Tie-2 receptor and their corresponding ligands vascular endothelial growth factor (VEGF-A) and angiopoietin-1/angiopoietin-2, respectively, play an important role in tumour angiogenesis and in haematological diseases [80]. Anti-VEGFR-2 intrabodies were described to block VEGFR-2 expression on the surface of porcine aortic endothelial cells and human umbilical vein endothelial cells (HUVECs) [41, 42]. The intrabodies inhibited *in vitro* angiogenesis. Interestingly, tumour angiogenesis was inhibited by intrabodies *in vivo* performed with human tumour xenograft mouse models. This was shown with an adenovirus-delivered anti-Tie-2 intrabody given to mice with well-established human Kaposi's sarcoma (SLK) or human colon carcinoma (SW1222) [43]. Furthermore, a new bispecific, tetravalent intradiabody was able to simultaneously knockout both cell surface receptors KDR and Tie-2 [44, 45]. Performed with a human melanoma xenograft mouse model, the intradiabody inhibited tumour growth by 92.2% [45]. In summary, it could be concluded that ER-targeted anti-VEGFR-2/KDR or anti-Tie-2 intrabodies might be useful for therapeutic application against solid tumours.

Another application of anti-oncogenic receptor ER intrabodies was shown with the human folate receptor [46]. The α -folate receptor (FR) is selectively overexpressed in 90% of non-mucinous ovarian carcinomas. Folate uptake induces signal transduction associated with dysregulated cell proliferation. Expression of anti-FR intrabodies in ovarian cancer cells led to a

dramatic decrease in cell proliferation rate and increased adhesion to various extracellular matrix components accompanied by reversion towards normal phenotype [46].

Last but not least, the ER intrabody technology was used to neutralize proteins involved in tumour development that do not belong to the growth factor receptor family. Intrabodies were generated that block the secretion of proteinases. This was shown for (i) procathepsin L that strongly increases the tumorigenicity and metastatic potential of human melanoma cells [81] and (ii) for the metalloproteinase MMP-2 and MMP-9 which both degrade collagenase IV in the basement membrane and play an important role in tumour invasion and formation of metastasis [82].

ER intrabodies against infectious diseases

ER intrabodies against HIV

To prevent the entry of HIV-1 into CD4⁺ T cells, the HIV-1 glycoprotein gp120 and the corresponding co-receptors can be targeted by neutralizing antibodies or small molecule antagonists. To inhibit different stages of HIV-1 life cycles, anti-HIV intrabodies have been targeted to structural, regulatory and enzymatic proteins of the virus inside the different compartments of the host cell [47]. The targets of anti-HIV ER intrabodies are the HIV-1 env glycoprotein gp120 [47, 48], the precursor gp160 [49] and the binding counterpart: the CD4 co-receptor CCR5 or CXCR4 [50–54].

Anti-HIV-1 gp120/gp160 intrabodies

An ER intrabody against the envelope protein gp120 led to inhibition of proteolytic processing of the env precursor gp160, decrease of envelope-mediated syncytium formation and reduced infectivity of HIV-1 particles released by intrabody expressing cells [47, 48]. Studies are evaluating the efficiency of the anti-gp120 intrabody in asymptomatic HIV-1-infected patients, by reinfusing autologous CD4⁺ T cells that have been transduced *ex vivo* with a murine leukaemia virus vector encoding this intrabody [47]. Furthermore, an scFv with an ER or trans-Golgi network (TGN) retention signal recognizing the envelope precursor gp160 inhibited HIV-1-induced syncytial formation too [49].

precursor gp160 inhibited HIV-1-induced syncytial formation too [49].

Anti-HIV-1 co-receptor intrabodies

HIV requires a co-receptor, in addition to CD4, for entry into target cells. Macrophage (M)-tropic viruses require the chemokine receptor CCR5 for entry, while T-cell-line (TCL)-tropic viruses use CXCR4 for entry. Intrabodies against CCR5 and CXCR4 led to protection against HIV-1 infection [50–54]. Interestingly, the anti-CCR5 intrabody [50] reacts with CCR5 from non-human primates and the strategy to establish an HIV-1 resistant cell pool in infected patients can be tested in SIV and chimeric simian-human immunodeficiency virus (SHIV) models of human AIDS. Recently the intrabody was transduced into CD34⁺ foetal liver stem cells and this cells gave rise to CD4⁺ and CD8⁺ thymocytes in non-obese diabetic (NOD)/severely combined-immunodeficient (SCID)-human thymus/liver (hu thy/liv) mice [51].

In another approach, the CCR5 surface expression was down-regulated in monocyte-derived macrophages after co-infection of Tag-deleted SV40-derived vector encoding an anti-CCR5 scFv fragment and a hammerhead CCR5-specific ribozyme. The combination of both transgenes prevented the cells from HIV-1 infection after challenge with high doses of HIV-1 [52]. In addition, recently a new anti-CXCR4 intrabody was described that inhibits infectious entry of HIV-1 in primary isolated human brain microvascular endothelial cells and post-mitotic differentiated human neurons [54]. This example opens the possibility to develop strategies to apply such intrabodies against HIV-1-related neurodegenerative disorders and neuroinvasion. In summary, the anti-envelope intrabodies might be used to generate cells that produce virions that are markedly less infectious, whereas the anti-CCR5/CXCR4 intrabodies might be used in clinical gene therapy strategies to generate a cell pool in infected patients that is protected from HIV-1 infection.

ER intrabodies to other viruses

A recombinant ER-targeted Fab fragment against the Hepatitis C virus core protein was described, and co-localization and intracellular binding was shown [83]. Two soluble scFv fragments against the

transmembrane envelope glycoprotein gp46 of maedi-visna virus (a retrovirus which causes pneumonia, encephalomyelitis and arthritis in sheep) recognized maedi-visna virus in the cytoplasm of fixed virus-infected sheep choroids plexus (SCP) cells [84]. These antibodies targeted to the ER as intrabodies could prevent the maturation process of the gp150 precursor envelope glycoprotein into gp135 and gp46 in virus-infected cells resulting in less infectious virus particles. Last, but not least, an ER intrabody was described that recognizes secretory salivary proteins of the root-knot nematode *Meloidogyne incognita* [85].

Abrogation of MHC I molecules

Presentation of intracellularly synthesized peptides in a complex with MHC class I molecules on the cell surface of donor cells is the central reason for rejection of allogeneic cell and tissue transplants. The generation of transplantable *in vitro* generated skin sheets for replacement of skin defects has become a standard procedure. Nevertheless, after transplantation the allogeneic MHC class I expressing keratinocytes are able to induce an alloimmune response *via* CD8⁺ T cells and infiltrating recipient APCs. Methods to down-regulate MHC I cell surface expression such as deletion of the MHC I associated protein β_2 microglobulin (β_2m) and deletion of the intracellular TAP (transporter associated with antigen processing) led to cells that still exhibit small amounts of MHC I molecules on the cell surface [86]. The intrabody technology represents an efficient promising approach to suppress allorecognition against MHC I. It could be shown that intrabody expression in primary rat and human keratinocytes prevented the cells from CTL-mediated lysis to a great extent [55, 56].

In another approach, surface MHC I molecules were declined in human umbilical vein endothelial cells (HUVECs) [57] and also in this case the intrabodies protect allogeneic HUVECs from CTL-mediated lysis. This might be important in the field of vascular surgery where transplantation of small-calibre vascular prosthesis seeded with autologous endothelial cells is performed. Following the studies with the keratinocytes and HUVECs, the ER intrabody strategy is a promising tool to avoid the allograft rejection induced by MHC I molecules of the host.

In addition, ER intrabodies against α 1,3-galactosyltransferase have been described [87]. The carbohydrate structure Gal α 1,3 Gal expressed on pig cells is the major antigen recognized by xenoreactive natural antibodies in higher primates. The expression of the anti- α 1,3-galactosyltransferase intrabodies in pig epithelial kidney cells inhibits almost completely complement dependent cytotoxicity mediated by purified anti-Gal antibodies. Nevertheless, it only partially reduced the cytotoxicity of whole serum due to non- α Gal xenoantigens recognized by human xenoreactive natural antibodies. The combination of the expression of such intrabodies with the expression of human regulators of complement activity and expression of enzymes that compete with α 1,3-galactosyltransferase for acceptor molecules might lead to a more efficient inhibition of xenogenic cytotoxicity.

ER intrabodies targeting integrins

The integrins are a large family of transmembrane glycoproteins that mediate cell–cell or cell–matrix interactions. In mammals, 24 integrins have been identified to date, resulting from different pairings among the 18 α - and 8 β -subunits. They play an important role in the regulation of cell survival, differentiation, proliferation, haemostasis and host defence. They bind to various extracellular matrix proteins such as fibronectin, vitronectin and osteopontin. In addition α V integrins play a role in the progression of cancer. Performed with an anti- α V integrin intrabody, new insights into the function of integrins during differentiation of osteosarcoma cells, in the regulation of cell surface expression of $\alpha_v\beta_1$ integrin and in the involvement of integrins during survival of metastatic cancer cells were obtained [58–60].

After transfection of an anti- α V integrin intrabody into osteosarcoma cells, the expression of the osteoblast differentiation marker genes, alkaline phosphatase and osteopontin, was induced whereas the expression of matrix metalloproteinase-2 was decreased [58]. These data indicated that α V integrins are important regulators of osteosarcoma cell phenotypes. The same group has also analysed whether the expression of integrin $\alpha_v\beta_1$ is selectively regulated by the hierarchical formation of other α V-containing heterodimers. They showed that trans-

fection of the same intrabody into melanoma cells resulted in the reduction of cell surface expression of only integrin $\alpha_v\beta_1$ [59]. This leads to the conclusion that the expression level of $\alpha_v\beta_1$ on the surface of melanoma cells is dependent on the number of α_v subunits available after the formation of other α_v -containing heterodimers. Furthermore, the authors constructed a recombinant adenovirus with the intrabody gene and used this adenovirus to express the intrabody in three metastatic melanoma cell lines. The adenovirus effectively inhibited the cell surface expression of α_v integrins and prevented tumour growth of one metastatic melanoma cell line in SCID mice [60].

ER intrabodies targeting proteins involved in Alzheimer's and Prion disease

Misfolded and accumulated intracellular proteins characterize a wide range of neurodegenerative disorders. Proteins involved in the Huntington's and Parkinson's diseases were successfully targeted by cytoplasmic and nucleus-targeted intrabodies [88] whereas ER intrabodies inhibited the cleavage or translocation of cell surface proteins from the ER to the cell surface that play an important role in the biogenesis of Alzheimer's and Prion diseases: β -APP [28] and PrP^C [61, 62].

Endoproteolysis of the β -APP by β - and γ -secretases generates the toxic amyloid β -peptide ($A\beta$), which accumulates in the brain of Alzheimer's disease (AD) patients. Generation of the toxic amyloid β -peptide was prevented by ER expression of an intrabody recognizing an epitope adjacent to the β -secretase cleavage site of the human β -APP [28]. In the case of Prion disease, SEKDEL-tagged anti-prion intrabodies were able to prevent abnormal scrapie isoform accumulation and inhibit prion infectivity in mice [61, 62]. These ER intrabodies are useful tools to study in more detail the cellular traffic and degradation of this important protein.

Intrabody gene delivery

The transfer of intrabody genes into living cells can be performed with viral or non-viral transfer systems.

Viral vectors

ER intrabody gene transfer was performed with adenovirus [34–37, 42–45, 56, 57, 60], murine leukaemia virus [32, 33, 39, 40, 47, 48, 50, 53], HIV-1-based lentivirus [30, 31, 51, 54], SV40-derived vector [52, 53, 89] and adeno-associated virus (AAV) [47]. Recombinant adenoviruses are the preferred vectors for gene therapy. The advantage of this DNA virus is the extremely efficient transduction of most tissues and dividing as well as quiescent cells and a high titre that can be produced. The expression of the transgene is transient and the viral genome does not normally integrate into the host genome with no risk for insertional mutagenesis. A significant problem associated with adeno-viral vectors is elicitation of an immune response against the vector and systemic delivery and is accompanied by rapid hepatic uptake of virus. Some improvements to extend plasma circulation of recombinant adenovirus have been reported [90]. Nevertheless, for *in vitro* transduction of ER intrabodies into cell lines [34, 35, 56, 60] and primary cells [42, 56, 57] and in mouse tumour models, recombinant adenovirus has been successfully employed [36, 43, 45, 60].

The advantage of lentiviral vectors is their capacity of integrating into non-dividing cells that constitute the targets of HIV-1 infection such as resting T cells, dendritic cells and macrophages and have been widely used in preclinical studies [91]. They can also infect proliferating cells and lentiviruses transfer genes to haematopoietic stem cells with a superior gene transfer efficiency. In addition, cells of the central nervous system have been successfully transduced.

Retroviral vectors are not able to transduce non-dividing post-mitotic cells. Further disadvantages are low vector titre and low transfection efficiency and particle instability. The retroviral vectors are suitable for *ex vivo* gene therapy, and despite the disadvantages, retroviral gene delivery systems have been used already in a number of clinical trials [92]. However, integration of retrovirus including lentivirus genomes into the host genome might induce oncogenesis in some clinical applications. The AAV, which is currently being tested in several human gene therapy trials, has several unique features that distinguish it from other gene therapy vectors: sustained transgene gene expression with low risk of insertional mutagenesis and the fact that host cellular immune responses have not yet been observed [93].

A major goal of gene delivery systems is the development of vectors that are able to target spe-

cific cell types. New approaches are transcriptional and transductional targeting [94–96]. Transcriptional targeting is generally achieved by placing the transgene under the control of a cell-type-specific promoter. Transductional targeting can be performed by pseudotyping (substitution of a part or all of the virus receptor with those from other virus strains or serotypes). Pseudotyping has been well established for lentiviral vectors. In this case, pseudotypes bearing glycoproteins of lyssavirus, lymphocytic choriomeningitis, alphavirus, filovirus, gammaretrovirus and baculovirus have been described [97]. Genetic fibre pseudotyping is performed to generate chimeric adenovirus with enhanced infectivity of ovarian cells without increasing gene delivery to murine livers [98]. Transductional targeting can also be carried out with bispecific molecular adaptors (usually bispecific antibodies) that simultaneously block native receptor binding and redirect the vector capsid to new cellular receptors or by genetically altering receptor-binding proteins in the virus capsid so that they bind to alternative receptors [94–96]. Performed with this approach, the normal receptor binding is abolished and/or a small peptide ligand for alternative receptor binding is incorporated into the capsid structure.

Adenovirus targeting was successfully performed in several cases [95]. In contrast to adenoviral vectors, envelope alterations of retroviral vectors including lentivirus affect in most cases viron assembly and lead to low fusion or defective fusion activity [96, 99]. With regard to the retargeting of lentivirus, two new reports performed with Sindbis pseudotyped lentiviral vectors were described displaying an anti-CCR5 scFv fragment [100] and an anti-P-glycoprotein antibody [101]. The modified lentivirus expressing the anti-P-glycoprotein antibody on the viron surface were successfully retargeted to P-glycoprotein on metastatic melanoma through intravenous injection of mice [101]. These two studies can be considered as a powerful approach for specific viral gene delivery mediated by Sindbis envelope that displays single-chain antibody fragments recognizing specific cellular surface proteins.

Non-viral transfer systems

Liposomes and other non-viral delivery systems (naked DNA, DNA-coated polymer, cationic peptide-

DNA complexes, metal-coated DNA) are under investigation for use in cancer gene therapy [102]. At present, the main disadvantage is the low transduction efficiency and transient transgene expression. Nevertheless, the delivery of genes by cationic liposomes is rather effective and used in worldwide human clinical trials of gene therapy. Recent studies combining structural and biological techniques are now unravelling the relationship between the distinctly structured cationic liposomes-DNA complexes and a new generation of liposomes is now in the process of evaluation [103].

Recent advances indicate that efficient, long-term gene expression can be maintained as small episomal plasmids or artificial chromosomes and integration of DNA can be targeted to specific genomic sites [102]. In addition, inclusion of ligands for receptor-mediated endocytosis, peptide sequences that enhance DNA compaction and endosomal disruption sequences enhances non-viral delivery to cells [102].

The use of cationic protein transduction domains (PTDs) that cargo peptides, proteins and nucleic acids into cells by a receptor-independent, fluid phase macropinocytosis was reported in different mouse models of cancer, ischaemia and inflammation [104]. These reports of successful *in vivo* transduction are exciting and encouraging. Nevertheless, a human scFv fragment recognizing the ED-B domain of fibronectin (a marker of the modified extracellular matrix associated with the tumour neovasculature) fused to the TAT peptide showed severely reduced tumour-targeting performance compared to the unconjugated antibody [105]. The properties of PTD-linked intrabody genes need to be examined in detail. In another promising approach, specific targeting of the therapeutic herpes simplex virus thymidine kinase gene was achieved with a so-called immunoportor consisting of a Fab fragment against EGF receptor conjugated with a cationic polylysine chain [106]. It was shown that the Fab immunoportor carrying the herpes TK gene was effective in preventing the growth of EGF receptor-overexpressing tumour cells.

Conclusions

The intrabody technology is a promising method to inactivate and to study specific protein functions

inside different compartments of a cell. It complements the RNAi and intramer technology and replaces the RNAi technology in cases in which the RNAi approaches fail due to unspecific effects. The advantage of ER intrabodies is that they are correctly folded in contrast to cytoplasmic expressed intrabodies. *In vitro*, they inhibit very efficiently the translocation of growth factor receptors from the ER to the cell surface, prevent HIV-1 virus infection and decrease the immunogenicity of allogenic tissue and transplants and target successfully proteins involved in the genesis of neurological disorders. However, until now they have not been applied for clinical applications in spite of some very promising results obtained from *in vivo* mouse models. A prerequisite for clinical approaches is the improvement of efficient cell- and tissue-specific intrabody gene transfer. Nevertheless, in the future ER intrabodies will be potent reagents to inactivate endosomally localized receptors involved in bacterial and viral infection diseases, intracellular oncogenic receptors and enzymes involved in glycosylation of tumour markers.

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