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Review article

Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy

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1. Introduction

Molecular techniques for inhibiting the expression of specific genes allow a highly refined approach to the analysis and manipulation of microbial and cellular pathways. Various strategies such as antisense RNA, ribozymes, dominant negative mutants and targeted gene disruption have all been successfully used to inhibit the expression or function of specific genes [1–4], but each have certain limitations. Trans-dominant negative mutants, for example, require a functionally inactive mutant which acts in a dominant manner to suppress the activity of the wild type protein. With antisense RNA approaches, the effects are sometimes incomplete or short lived and may be frustrated by endogenous unwindase activities [1].

Intracellular antibodies, hereafter called 'intrabodies', that are synthesized by the cell and targeted to specific cellular compartments represent the most recent innovation in this field, and have been used to inactivate proteins in the endoplasmic reticulum (ER), cytoplasm and nucleus [5–7]. Endogenous synthesis of the antibodies has allowed a more precise method of intracellular delivery than had earlier methods that involved microinjection [8–10], pinocytotic uptake [11], polymeric-immunoglobulin receptor-mediated uptake [12], or enhanced endocytosis after cationization [13] of preformed antibodies into cells.

Early molecular studies had demonstrated that coexpression of cytoplasmic heavy and light chains of a neutralizing anti-alcohol dehydrogenase I monoclonal antibody in *Saccharomyces cerevisiae*, could provide limited neutralization of enzyme activity in vivo [14]. Similar studies were extended later to demonstrate that a catalytic antibody with modest chorismate mutase (EC 5.4.99.5) activity could function inside a yeast cell lacking the natural enzyme, and confer a growth advantage to its host by virtue of its catalytic activity [15]. Secretory, cytoplasmic and nuclear expression of immunoglobulin heavy and light chains had also been demonstrated in mammalian cells [16–18]. However, further structural improvements such as in single chain design and stable expression, were required for the full power of this technology to

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inhibit functions in mammalian cells to be demonstrated [5,6].

Recent advances in antibody engineering have allowed antibody genes to be manipulated and antibody molecules to be reshaped [6,19]. By harvesting the genetic information of the immune system in the form of rearranged immunoglobulin genes, intrabodies of high affinity and fine specificity can be created. These technological advances, combined with the wealth of information that has been obtained on classical intracellular protein trafficking signals [20], has allowed intrabodies to be directed to and stably expressed in many different subcellular compartments, an advantage over inhibitory RNA-based strategies. The marriage between these two disciplines has created a new and powerful research tool to analyze and manipulate microbial and cellular proteins. When combined with the rapid advances being made in gene transfer technologies [21–25], the clinical gene therapy application of the intrabody technology should be rapidly realized.

This review will concentrate on advances that have been made in the expression of intrabodies in animal cells. Antibody expression in plant cells has been reported using similar technologies [26–28]. There are several excellent reviews on antibody engineering [29–31] and gene therapy of HIV-1 infection [32–36], so these topics will not be addressed.

2. Defining terms

As this field is rapidly developing, it will be useful to define some basic nomenclature. Nomenclature used in this article:

- intrabody, intracellular antibody of any form;
- sFv, single-chain variable region fragment;
- sFv-fusion protein, single-chain variable region fragment-fusion protein;
- Fab, monovalent antigen binding fragment.

3. Starting materials for intrabody construction

The intrabodies that have been reported to date are derived from murine monoclonal [7,37–45] or human monoclonal antibodies [6,46,47]. In the absence of a suitable hybridoma, however, synthetic intrabodies can readily be generated using recent antibody engineering advances including phage display technology [48–51], repertoire cloning [52–54], in vitro affinity maturation [55,56], semi-synthetic libraries [57–59], very large library creation [60] and guided selection [61]. These recombinant DNA methods to generate intrabodies de novo will eliminate some of the problems that result from using hybridomas as starting materials for intrabody construction, including the variable presence of multiple light chain transcripts [62,63]. Starting materials for these recombinant DNA-based strategies have included RNA from mouse spleens [52] and human peripheral blood lymphocytes [49,53], lymphoid organs and bone marrow from HIV-1-infected donors [64,65].

4. Intrabodies against the human immunodeficiency virus

Intrabodies may be especially well suited to target viral and cellular proteins trafficking through the secretory system. In theory, there are multiple points within the secretory pathway at which an intrabody could be placed to bind and divert a trafficking protein from its ultimate destination. The ER may be the most strategic location, as the tubular architecture of this organelle, combined with the precise channeling of proteins through the secretory pathway, should maximize the chances of interaction between a resident intrabody and the target protein. Unlike more distal parts of the secretory system, peptide signals required for the ER-retention of soluble proteins are well characterized and consist of the carboxy terminal tetrapeptide Lys-Asp-Glu-Leu (KDEL) [66]. The ER is also the natural site of antibody assembly, being residence to molecular chaperones such as BiP and GRP94, which assist in the correct folding of immunoglobulin molecules [67]. Lastly ER-resident proteins often show extended half lives.

4.1. sFv intrabodies against HIV-1 envelope glycoprotein targeted to the endoplasmic reticulum

The envelope protein of HIV-1 is located on the exterior of the mature virus particle. The glycosylated envelope protein precursor gp160 is

translocated into the ER after synthesis, and is cleaved within the Golgi apparatus to yield a mature envelope protein composed of gp120 and gp41 [68] (Fig. 1). The human monoclonal antibody that was engineered for these experiments was F105 which competes with CD4 for binding to gp120 [69,70]. The strategy was to design an sFv intrabody that would be directed to the lumen of the ER, where it could bind gp160 precursor and prevent its transport to the cell surface (Fig. 2). For these studies, two different sFv105 intabodies were designed which differed only by the presence of a C-terminal KDEL sequence. This sequence is predicted to cause the retention of soluble proteins in the lumen of the ER [66]. Unexpectedly, sFv105 which was designed for secretion was stably retained in the ER, whereas the sFv105KDEL intrabody yielded an unstable protein that was rapidly degraded if not coexpressed with envelope glycoprotein [6]. The sFv105 intrabody had greater inhibitory effects in all assays tested compared to sFv105KDEL. The results demonstrated that the sFv105 and envelope glycoprotein coprecipitated and that; (1) proteolytic processing of the envelope precursor was inhibited, (2) cytopathic effects of the envelope protein were reduced, in that envelope-mediated syncytium formation, was markedly reduced in assays with $CD4^+$ cells, and (3) the infectivity of HIV-1 particles released by sFv105 expressing cells was substantially reduced.

An inducible expression vector was also constructed in which sFv105 was under the control of the HIV-1 long terminal repeat (LTR)/promoter and stable human $CD4^+$ lymphocyte cell lines were established [46]. In these studies, the sFv105 intrabody was inducibly expressed after HIV-1 infection, or in the presence of exogenous Tat protein (which is taken up by the cells and binds to the HIV LTR promoter to transactivate viral gene expression (see below)), and was retained intracellularly. The cells stably transfected with this expression vector exhibited resistance to the virus-mediated syncytium formation, and a decreased ability to support HIV-1 production. In addition, whereas in contrast to cells transfected with control vector in which surface CD4 was down-regulated following HIV-1-infection (association of gp160/$CD4^+$ in the lumen of the ER is known to cause down-regulation of surface CD4 [71–73]), in the sFv105 expressing HIV-1-infected cells, surface gp120 expression was markedly reduced and surface CD4 was normal. These results demonstrated that intracellular protein-protein interactions could be inhibited by the sFv intrabodies. Cell surface phenotype, replication rate, morphology, and response to mitogenic stimulation of the transformed cells were also normal.

To investigate the mechanism(s) whereby sFv105 was intracellularly retained (in the absence of the KDEL retention signal), coimmunoprecipitation experiments were performed with a rat monoclonal antibody to the ER chaperone protein, BiP (GRP78) [74]. Indeed, sFv105 was coimmunoprecipitated with BiP and anti-BiP [6]. From these experiments, we concluded that retention of sFv105 was probably a consequence of association with BiP, however, binding to BiP did not impair the specific binding activity of sFv105. Normal immunoglobulin heavy or light chains expressed in the absence of the appropriate counterpart light or heavy chains also bind to BiP, and can be retained in the ER. These normal proteins are not exported until the correct partner is introduced [74–77]. Other ER chaperone proteins including GRP94 [67,78] and IP90 [79], that are known to associate with unassembled immunoglobulin chains may also cause the ER retention of some sFv intrabodies, perhaps due to their unusual folding. In addition, point mutations in the framework of a V_L, similar to a point mutation found in the F105 light-chain framework, has been shown to result in ER retention of the light chain [70,75,76]. In these studies, the defect in secretion of the light chain was not due to misfolding of the light chains, as the light chain assembled into functional antibodies when coexpressed with an immunoglobulin heavy chain. Variable secretion in eukaryotic cells of ER-directed sFv intrabodies which lack a KDEL sequence has also been reported by others [41,45,80].

4.2. Fab intrabodies against HIV-1 envelope glycoprotein targeted through the secretory pathway

Although in the previous studies we demonstrated that sFv105 was retained in the ER and could block transport of the envelope glycoprotein and production of infectious HIV-1 virus, this

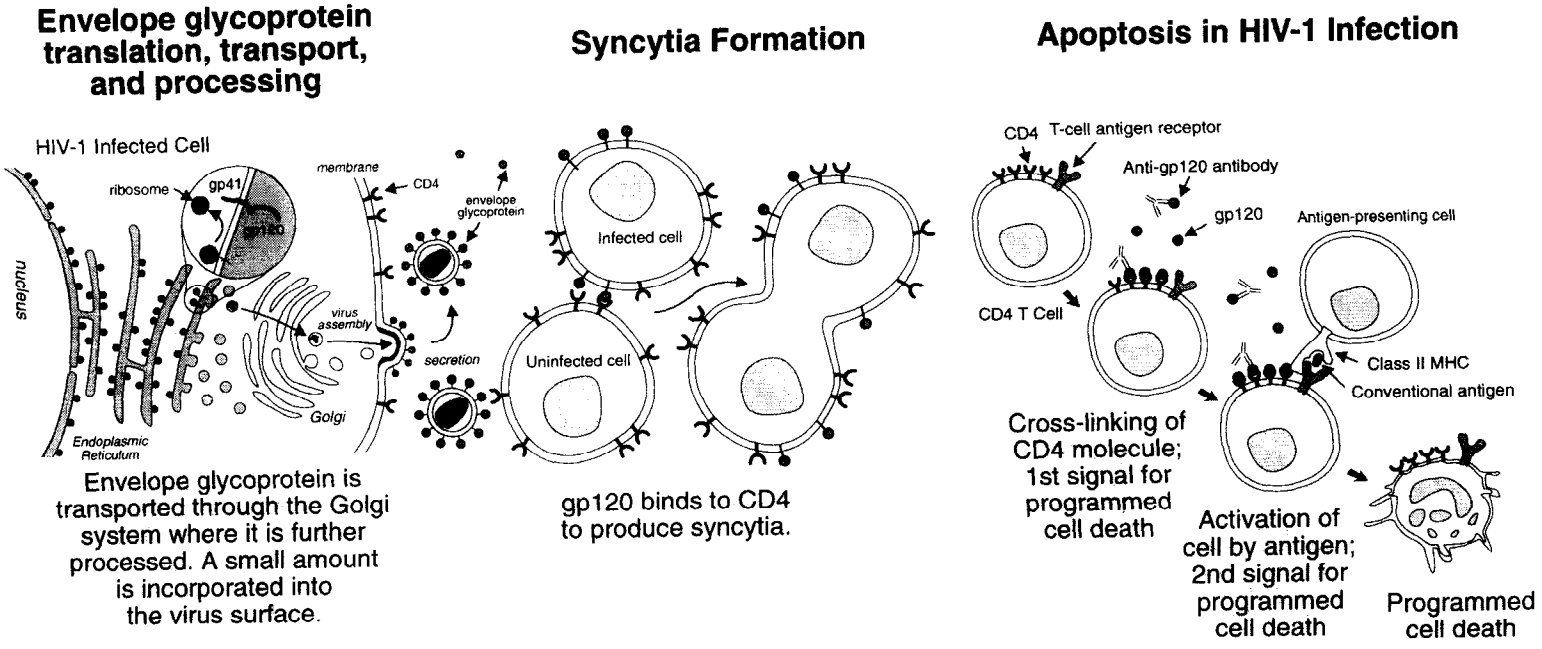

Fig. 1. HIV-1 envelope processing, transport and associated cytopathic processes of syncytia formation and apoptosis. Apoptosis is proposed to occur by either soluble gp120 or anti-gp120/gp120 complexes binding to CD4 prior to (instead of simultaneous with) engagement of the T cell receptor/antigen/MHC II complex.

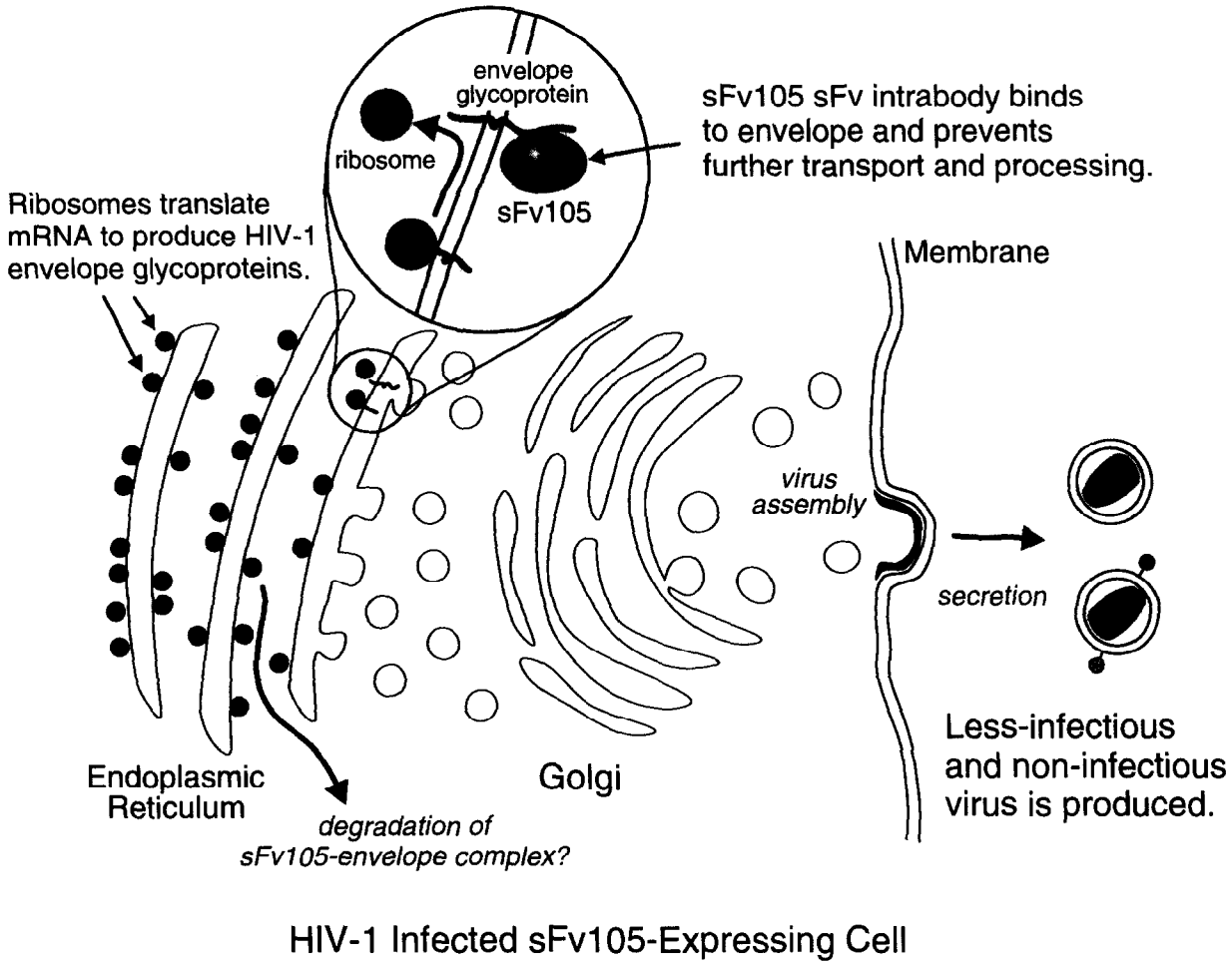

Fig. 2. Inhibition of gp160 precursor processing in sFv105 expressing cells leads to inhibition of envelope transport and the release of less-infectious and non-infectious virions.

interaction could only occur in the ER, and did not allow a mechanism for intrabody binding to any gp120 that had been transported beyond the ER. To accomplish this, the rearranged F105 heavy and light chain genes were used to construct a vector in which the heavy and light chains of a Fab fragment (Fab105) could be simultaneously expressed and secreted. Stable human $CD4^+$ T lymphocyte cell lines that would secrete Fab105 intrabody fragments were established [47]. This strategy has the additional advantage of allowing the transduced cells to secrete broadly neutralizing Fab105 fragments that would bind to cell free virus and prevent infection of surrounding non-transduced cells (Fig. 3). In the transduced cells infected with HIV-1, the nascent Fab105 fragments bound intracellularly to the HIV-1 envelope protein precursor gp160, and inhibited processing to gp120, and infectious HIV-1 production. The secreted Fab105 fragments were also able to neutralize cell-free HIV-1. In addition, the nascent Fab105 intrabody fragments could inhibit HIV-1 production by binding intracellularly to envelope mutants that escape neutralization by extracellular F105 antibody. This remarkable finding suggested that the high concentrations of Fab105 fragments

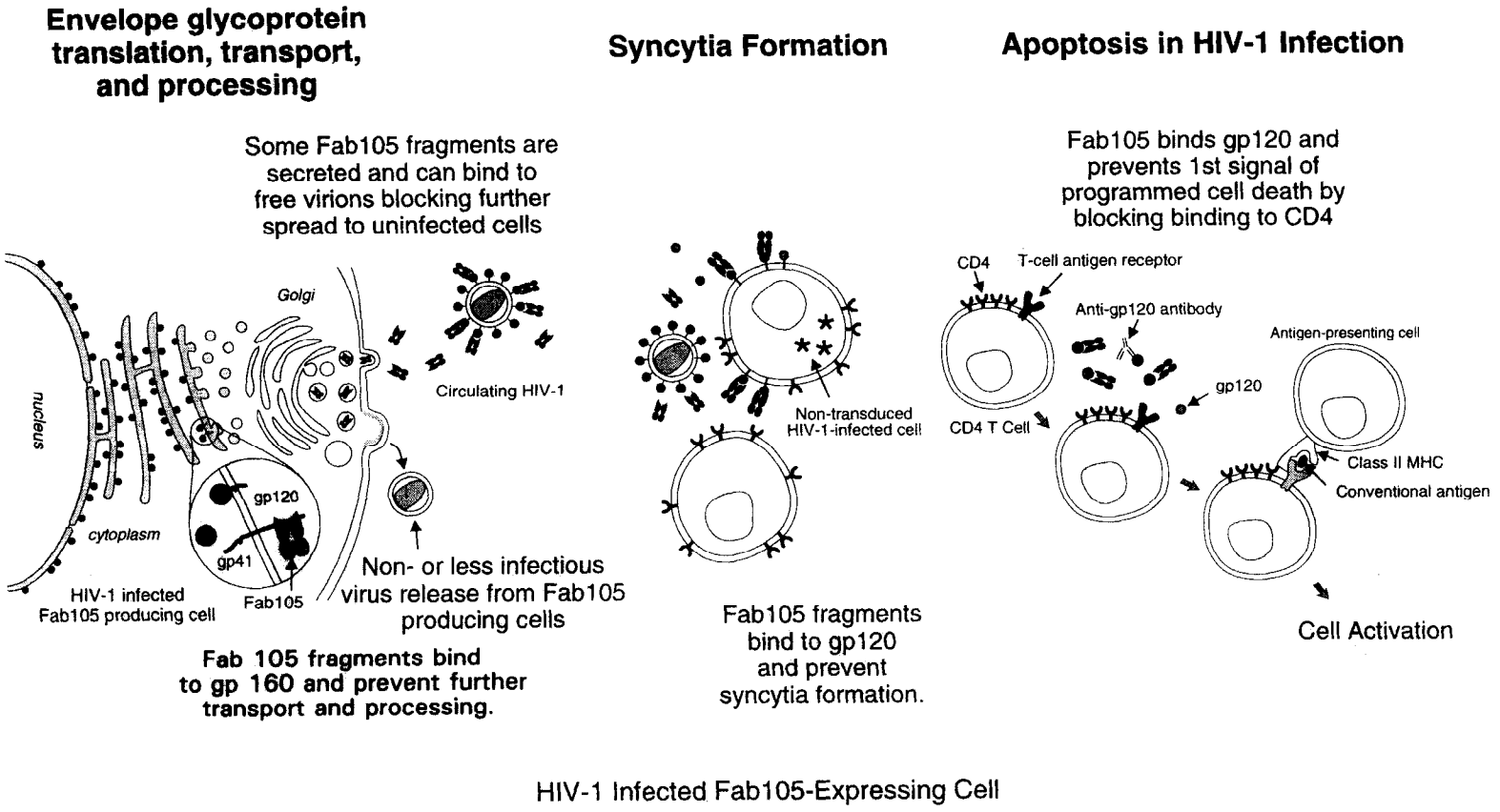

Fig. 3. Combined intra- and extra-cellular immunization by Fab105 expressing cells.

in the secretory vesicles could compensate for decreases in affinity that resulted from point mutations in the envelope glycoprotein. Similar observations have been reported with lower affinity CD4 mutants that are retained intracellularly [81].

In summary, the strategy of using anti-env intrabodies to transduce $CD4^+$ peripheral blood T lymphocytes or $CD34^+$ stem cell in a clinical gene therapy setting, should result in the production of virions that are markedly less infectious. As a consequence, the spread of HIV-1 in an HIV-1-infected patient may be reduced. In addition, since the interaction of gp120-CD4 either on the cell surface or intracellularly is crucial not only for virus infection, but also for many of the postulated mechanisms of $CD4^+$ depletion and functional abnormalities such as synctium formation, induction of apoptosis or single-cell lysis, disruption of CD4-mediated cell signalling and inappropriate immune responses [82], this gene therapy approach may provide a therapeutic benefit for HIV-1-infected patients by several different mechanisms [6,46,47]. Moreover, the studies also demonstrated that the Fab105 intrabody transduced T lymphocytes were not only resistant to HIV-1 infection by intracellular binding activity of nascent Fab105 intrabodies, but also were able to protect surrounding lymphocytes by secreting neutralizing Fab105 fragments.

4.3. Inactivation of cytoplasmic and nuclear HIV-1 regulatory proteins Tat and Rev using sFv intrabodies and their modified forms

The expression and anti-HIV-1 inhibitory activity of sFv intrabodies directed against the critical HIV-1 regulatory proteins Tat and Rev have been reported [7,39,40]. Tat is a small one (72-amino acid) or two (86-amino acid) exon-derived nonstructural protein that is known to be a potent transcriptional activator of HIV-1 long terminal repeat (LTR) [83–85]. The sequence in the 5′ untranslated region of all HIV RNAs called transactivation response element (TAR) (nucleotides +1 to +44), is required for the Tat-mediated transactivation [86]. TAR RNA forms a stable stem-loop structure and Tat binds to its bulge region with high efficiency [87] (Fig. 4). This RNA-protein interaction is essential for Tat activation of the HIV-1 LTR [88]. Tat functions primarily to stimulate transcription initiation and to increase transcription elongation [84,89,90]. This Tat-mediated stimulation of transcription involves an interaction between Tat and other Tat-binding cellular proteins, some of which may be transcription factors themselves [91–93], as well as cellular proteins bound to the TAR region [85,94–96].

To construct anti-Tat sFv intrabodies that were capable of binding Tat in the cytoplasm or nucleus of eukaryotic cells, genes encoding the rearranged immunoglobulin heavy and light chain variable regions of anti-HIV-1 Tat, exon1- or exon2-specific monoclonal antibodies were used to construct sFv intrabodies. The idea here was, that if anti-Tat sFvs could bind to Tat, subsequent inhibition of Tat-mediated transactivation of viral mRNA transcription could occur (Fig. 4). Accordingly, several anti-Tat sFv expressor vectors were constructed to target the anti-Tat sFvs either to the cytoplasmic or nuclear compartments. To ensure a high level of expression, the coding sequences of all anti-Tat sFvs were modified to contain a Kozak consensus sequence and start methionine immediately 5′ to amino acid one of framework one of the heavy chain [97]. For each parent anti-Tat sFv, an additional anti-Tat sFv derivative (sFv-fusion protein) was constructed for cytoplasmic expression that had the entire human κ chain constant domain (C_κ) fused in frame at the carboxy-terminus of the sFv cassette (sFvtat-C_κ), and was designed to increase the stability and/or possibly promote dimerization in a form similar to Bence-Jones proteins [98]. For nuclear targeting, the sFvtat and sFvtat-C_κ were additionally modified to contain the carboxy-terminal SV40 nuclear localization signal (sFvtat-SV40 and sFvtat-C_κSV40, respectively) which has been shown to direct heterologous proteins into the nucleus [99]. All anti-Tat sFvs were cloned under the control of the human cytomegalovirus immediate early promoter (CMVIE). Several important specific and general results were obtained. First, the anti-Tat sFvs with specific binding activity against the N-terminal activation domain of Tat were able to block Tat-mediated transactivation of HIV-1 LTR (using a CAT reporter gene), as well as intracellular trafficking of Tat in mammalian cells. This in-

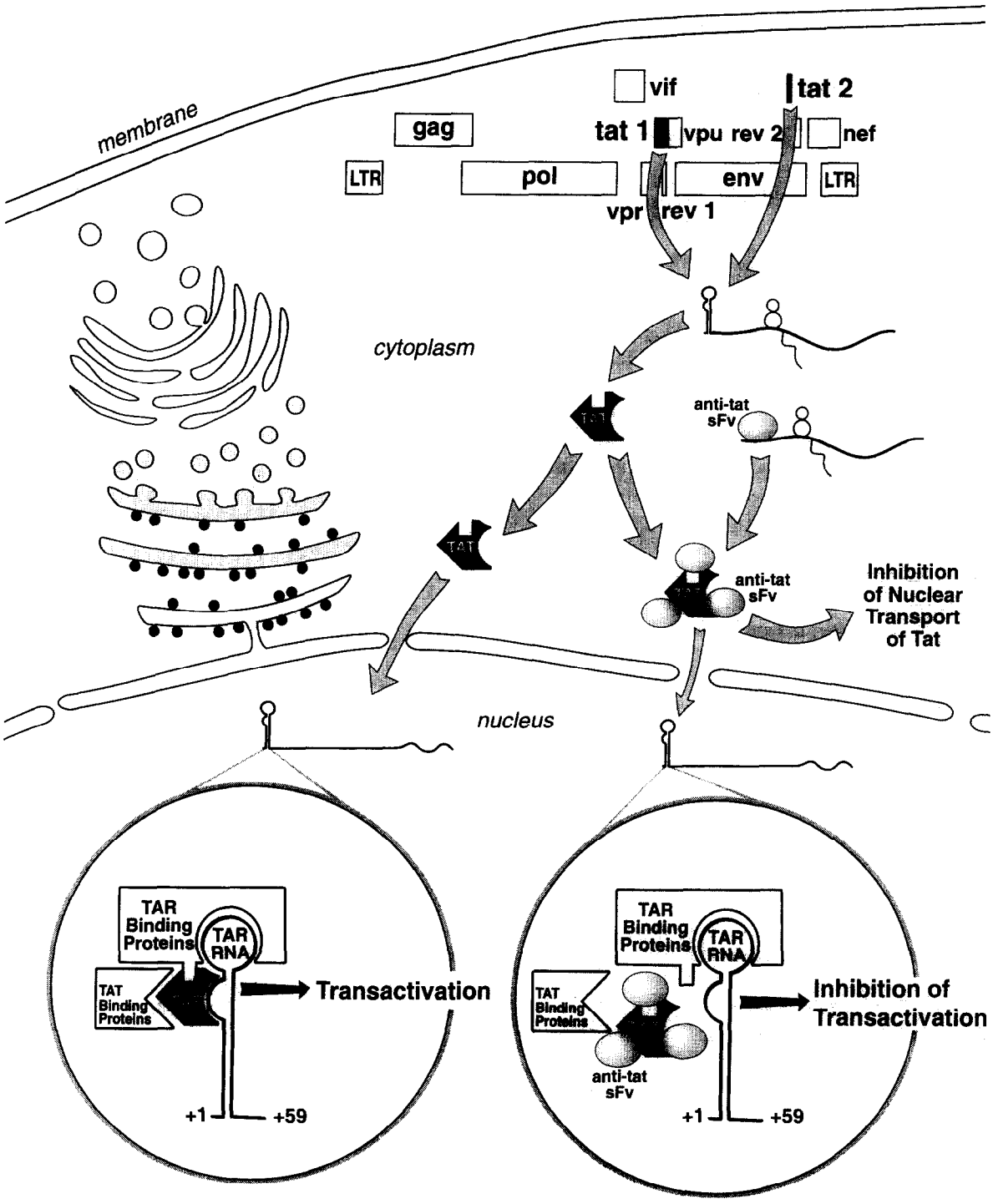

Fig. 4. Strategy for inhibition of Tat-mediated transactivation of viral gene expression by anti-Tat sFv intrabodies. Left lower insert: Tat binding to TAR RNA and other cellular factors are required for transactivation. Right lower insert: anti-Tat sFv binding to any of several critical epitopes on Tat may inhibit transactivation by inhibition of Tat nuclear import or by inhibition of Tat interactions in the nucleus with cellular factors and/or TAR RNA.

hibition occured despite the fact that Tat is present in very low concentrations in HIV-1-infected cells. As a result, the transformed lymphocytes expressing anti-Tat sFvs were resistant to HIV-1 infection. Second, nuclear targeting of the anti-Tat sFv was not required to inhibit the function of Tat (which acts in the nucleus). This result suggested that by inhibiting nuclear import of Tat, Tat/TAR RNA interactions in the nuclear compartment could effectively be inhibited. Third, in transfected $CD4^+$ T lymphocytic cells, sFvtat-C_κ fusion proteins had greater inhibitory activity than their sFvtat counterparts, which suggested that the addition of the C_κ domain resulted in increased stability of the sFv intrabody and/or possibly dimerization [98]; and fourth, stably expressed sFv intrabodies and their modified forms could effectively target molecules in the cytoplasm and nuclear compartments of eukaryotic cells, and that folding of the sFv intrabodies to form a functional binding site could occur in the reducing environment of the cytoplasm. The efficiency of this folding process is unknown.

Other investigators have targeted the critically important HIV-1 Rev protein. Rev is another small 116-amino acid, 20-kDa HIV-1 regulatory protein that is the regulator of virion protein expression [100]. Rev is encoded by multiply spliced viral mRNA and appears to efficiently function only in multimeric form [101,102]. The Rev protein controls virion protein expression by controlling the cytoplasmic accumulation of RNA species (Fig. 5). In the absence of an active Rev protein, only spliced mRNAs can be transported to the cytoplasm. The sequences coding for the capsid, replicative enzymes, and envelope glycoproteins have been removed by splicing. In the presence of Rev binding to the *cis*-acting Rev-responsive element, designated RRE, that is present in unspliced (genomic and *gag/pol* mRNA) and singly spliced (env mRNA) viral RNA, cytoplasmic transport of unspliced and singly spliced viral mRNA occurs, ultimately allowing HIV-1 replication [100]. In the studies by Duan et al. [39,40], a cytoplasmic intrabody against Rev was constructed and stably transduced into HeLa cell lines. In these anti-Rev sFv intrabody expressing cells, nuclear transport of Rev, syncytia formation and HIV-1-production were inhibited [39,40]. Moreover, no alterations in HIV-1 internalization, reverse transcription, or initial transcription of multiply spliced viral mRNAs were demonstrated despite inhibition of HIV-1 replication [40]. These results imply that the anti-Rev sFv intrabody is working in a manner similar to sFvtat [7,103], namely, by binding Rev in the cytoplasm and preventing its nuclear translocation and possibly its proposed nucleocytoplasmic shuttling activities [104–107]. As a result, subsequent interaction of Rev with cellular Rev binding proteins [104,108,109], or with the RRE containing viral mRNA and associated RRE binding proteins [110,111] is inhibited.

4.4. Combined intrabodies for gene therapy of HIV-1-infection

Collectively, these studies demonstrate that anti-HIV-1 intrabodies can be directed to multiple HIV-1 target proteins that are present in different subcellular compartments in the cell including the ER [6,46], secretory pathway [47], cytoplasm [7,39,40] and nucleus [7]. These studies also suggest that different anti-HIV-1 intrabodies, used either alone or in combination with other intrabodies or other genetic based strategies, may be useful for the gene therapy of HIV-1 infection and AIDS. The idea of using a multi-targeted approach for AIDS treatment has gained considerable ground in the last few years because of the failure of many single agents to halt the spread of HIV-1 [7,112].

We have created a bicistronic expression vector that is capable of expressing two different anti-HIV sFv intrabodies or the heavy and light chains of the Fab intrabodies, simultaneously (Fig. 6). The bicistronic cassette can be easily moved into efficient gene transfer vectors for primary cell gene transfer. An internal ribosomal entry site (IRES sequence) corresponding to the 5′ untranslated terminal repeat (UTR) of the encephalomyocarditis virus (EMCV) [113,114], has been cloned into the vector between two open reading frames to effectively create a bicistronic transcription unit. The IRES sequences allow cap-independent ribosomal binding and translation which often results in as efficient translation of the second protein as the first protein [115–119]. For example, for the

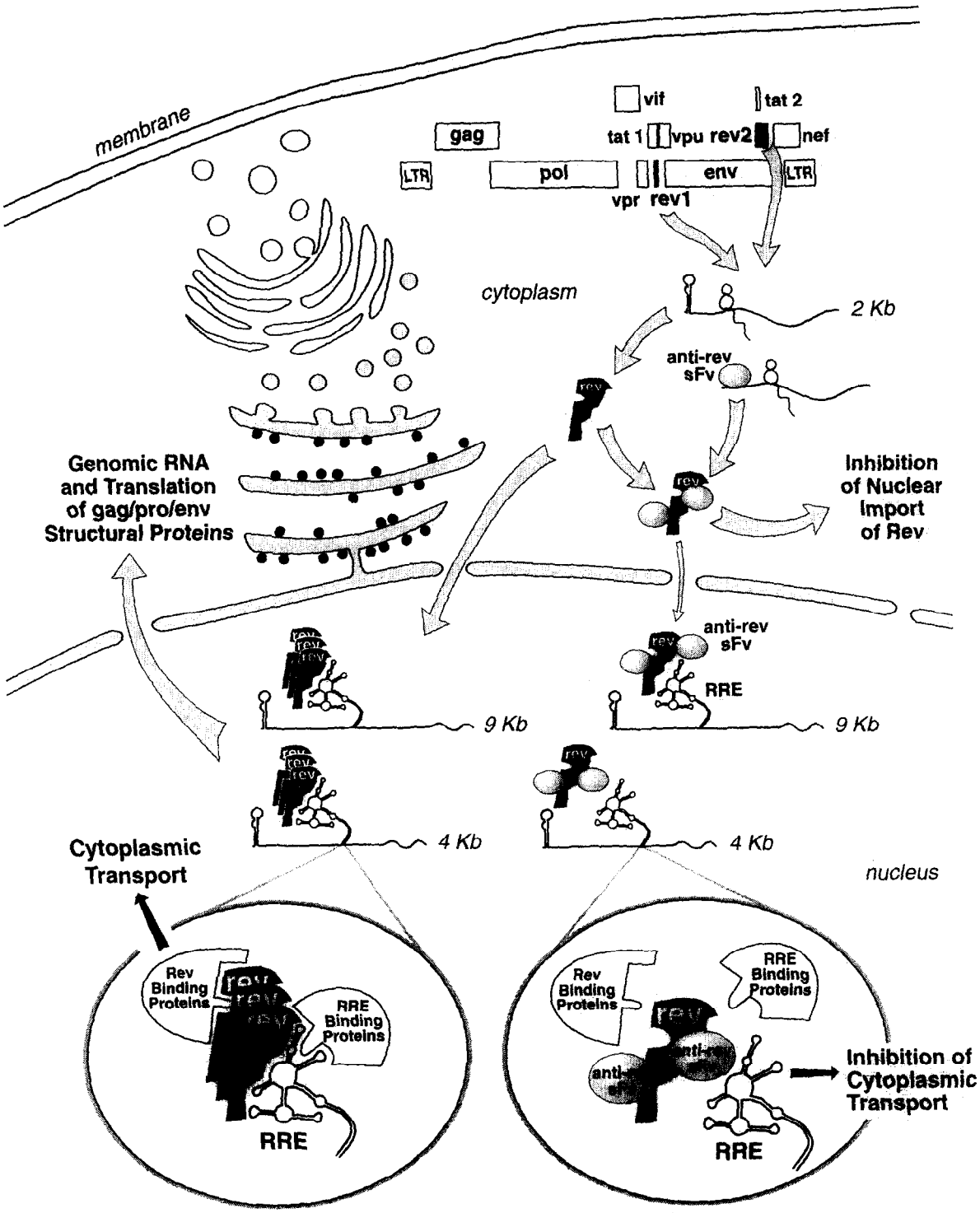

Fig. 5. Strategy for inhibition of Rev-mediated cytoplasmic transport of intron containing viral mRNAs. Left lower insert: Rev binding to the *cis*-acting RRE sequence in unspliced and singly spliced viral mRNA leads to cytoplasmic RNA transport, viral structural protein synthesis, virus assembly and production of infectious virions. Right lower insert: anti-Rev sFv binding to any of several critical epitopes on Rev may inhibit Rev nuclear import and/or inhibit Rev interactions in the nucleus with cellular factors and/or RRE containing viral mRNA.

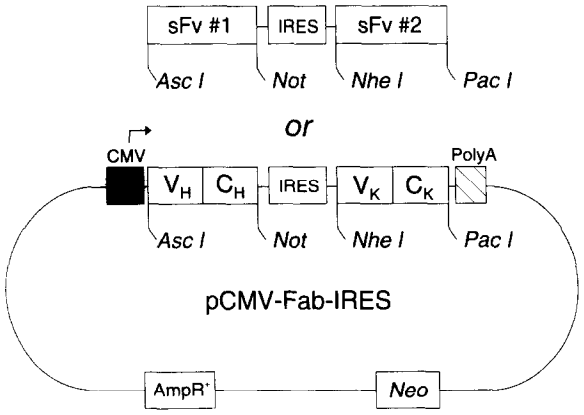

Fig. 6. Bicistronic expression vector pCMV-Fab-IRES. An internal ribosomal entry site (IRES), the 5′ UTR of the encephalomyocarditis virus (EMCV), has been cloned into the vector. Unique restriction enzyme sites allow the cloning of either heavy and light chains of Fab intrabodies or two sFv intrabodies.

secretion of the two-chain Fab105 intrabody fragments, the light chain was translated by ribosomal recognition of the EMCV IRES sequence, and this resulted in the expression of stoichiometric equivalent amounts of light chains, as compared to the heavy chains that were driven off the CMV promoter (unpublished observations). Thus, using this expression vector, we are capable of cloning and testing different combinations of intrabodies for their combined or synergistic inhibition of HIV-1 replication.

4.5. Using anti-HIV-1 intrabodies to target early events of the HIV-1 life cycle

To inhibit HIV-1 spread in a gene therapy setting it would be optimal if anti-HIV-1 intrabodies could be used to prevent infection, rather than limiting virus production by acutely and chronically HIV-1-infected cells [120–122]. Indeed, earlier events of the HIV-1 life cycle may be targetable using anti-HIV-1 intrabodies to prevent infection of susceptible cells. For example, virus uncoating may be disrupted by using intrabodies directed against mature *gag* products, including p17(MA) which forms an envelope-associated outer shell of the mature virion [123], or p24(CA) which forms the shell of the cone-shaped core structure which surrounds a complex of genomic RNA and viral *gag*- and *pol*-encoded proteins [123,124]. Well characterized monoclonal antibodies against these [125,126] and other *gag* proteins (p7(NC) and p6) have been reported [126,127]. Intrabodies that are directed to these mature *gag* proteins may also have the dual ability to interfere with virus assembly [128,129] and/or with cellular protein association with the maturing viral particle, such as the association of p24 and cyclophilin [130,131].

Recent studies have identified components of the pre-integration complex to include p17(MA), integrase and reverse transcriptase [132–134]. Nuclear localization signals on p17(MA) [135,136] and vpr [137] have also been identified, which appear to allow the establishment of HIV-1-infection in macrophages and quiescent T lymphocytes where the nuclear membrane is intact. Anti-HIV-1 intrabodies that are directed against these nuclear localization signals or against the active sites of integrase or reverse transcriptase, may provide another avenue by which these intrabodies may prevent integration by blocking the nuclear transport or disabling the pre-integration complex. If these HIV-1 protein targets (while part of the pre-integration complex) prove accessible to the intrabodies, then HIV-1-infection may be aborted and true 'intracellular immunization' may be achieved.

In summary, these studies suggest that anti-HIV-1 intrabodies used either alone or in combination and/or with other genetic based strategies may be useful for the gene therapy of HIV-1 infection and AIDS. One obvious requirement for this strategy to be successful is that high levels of intrabody expression can be achieved in transduced primary blood $CD4^+$ lymphocytes and/or $CD34^+$ stem cells, and that protection can be demonstrated against primary HIV-1 isolates. The gene transfer vectors will have to allow stable integration to occur in order to achieve long-term gene expression. Currently, MMLV-based retroviral vectors and adeno-associated-virus (AAV) vectors show the greatest promise to achieve stable and long-term expression of the intrabodies (unpublished observations), however, rapid advances in other viral [23,25] and non-viral gene transfer systems [24,138] may change the arena.

5. Growth hormone receptors

IL-2Rα is a growth factor receptor of clinical importance and is involved in the stimulated growth and effector functions in a variety of cell types including T and B lymphocytes, monocytes, lymphokine-activated killer cells, and natural killer cells [139–142]. IL-2Rα is also overexpressed in a variety of T and B cell leukemias, most notably in HTLV-1-associated adult T cell leukemias [143]. The high affinity IL-2 receptor is a heterotrimer composed of α, β and γ chains [142,144] (Fig. 7). The β and γ chains (IL-2Rβ and γ_c) form a receptor of intermediate affinity [144], which is present on resting T cells [145]. Expression of the 55-kDa α chain (IL-2Rα, Tac), and hence the high affinity receptor, occurs only transiently following engagement of the T cell receptor and is a key point of regulation in the T cell response to antigen [146].

Recently, we have described the use of two intrabodies to inhibit the cell surface expression of IL-2Rα [45]. For these studies, the anti-Tac hybridoma [147] was used as the source of RNA encoding the monoclonal antibody. We compared the activities of two sFv intrabodies: sFvTac and sFvTacKDEL, which differ only by the presence of an ER retention signal on the latter. Multiple aspects of intrabody behavior, including the stability, efficacy, binding specificity and intracellular fate of the two proteins were affected by the presence or absence of an ER retention signal. Most significantly, the ER-resident intrabody was significantly more effective at blocking the cell surface transport of IL-2Rα. Several lines of evidence pointed to the ER intrabody operating in precisely the expected manner, i.e., by binding and holding the receptor chain in the ER. First, the sFvTacKDEL was coprecipitated with IL-2Rα suggesting a physical interaction between the two proteins inside the cell. Second, IL-2Rα was detectable with these cells as an immature 40-kDa form that was sensitive to endoglycosidase-H, consistent with its retention in an early or pre-golgi compartment. The sFvTac intrabody was also able to downregulate IL-2Rα but, unlike sFvTacKDEL, gave a 'leaky' phenotype characterized by low level expression of IL-2Rα at the cell surface. In contrast to the stable complexes formed between the IL-2Rα and sFvTacKDEL, IL-2Rα complexed to sFvTac appeared to undergo rapid degradation within the ER. The degradation appeared to be via a non-lysosomal mechanism as the presence of methionine methyl ester, a lysosomal inhibitor, did not result in accumulation of IL-2Rα/sFvTac complexes in these cells. From these studies we concluded that the sFvTac and sFvTacKDEL intrabodies will provide a valuable tool for examining the role IL-2Rα in T cell activation, IL-2 signal transduction and the deregulated growth of leukemic cells which overexpress IL-2Rα.

The biological effects of an ER-directed anti-human epidermal growth factor receptor (EGFR) sFv intrabody without (scFv225S) and with the KDEL retention signal (scFv225R), were evaluated in EGF receptor-transformed NIH/3T3 cells [41]. The inhibitory effects of the KDEL minus sFv intrabody compared to the KDEL plus sFv intrabody on EGFR protein expression, EGFR-induced activation of cellular phosphotyrosine content, and EGF-dependent colony formation in soft agar, although modest, were greater for the cells expressing the KDEL minus sFv intrabody compared to the KDEL plus sFv intrabody, leading the authors to conclude that the KDEL minus sFv intrabody functioned in an autocrine fashion to inhibit the activity of the EGF receptor.

6. Oncoproteins

Intracellular expression of sFv intrabodies directed against the extracellular domain of erbB-2 have been demonstrated to revert erbB-2 transformation [42]. The erbB-2 gene encodes a 185-kDa transmembrane glycoprotein that is a member of the subclass I, EGFR-related tyrosine kinases [148–151]. The oncogenic potential of the erbB-2 protein can be activated by different mechanisms including point mutation and overexpression [152,153]. Amplification and/or overexpression of erbB-2 is observed in tumors arising at many sites including, breast and ovary where it correlates with an unfavorable patient prognosis [154–156]. Beerli et al. [42] reported on the biological effects of anti-erbB-2 sFv intrabodies that bind to the extracellular domain of erbB-2 and that were

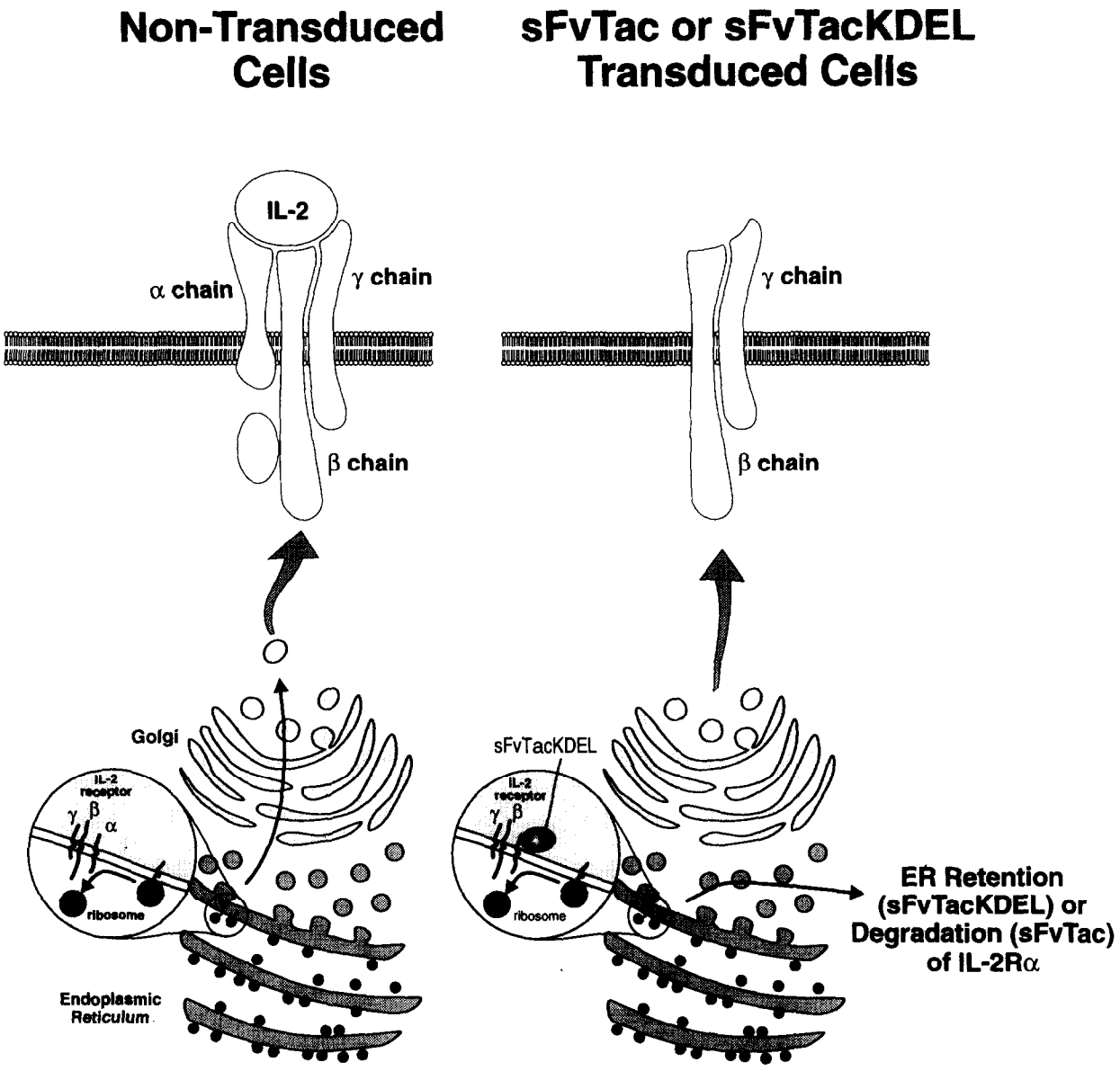

Fig. 7. Phenotypic 'knockout' of IL-2Rα. Expression of anti-IL-2Rα sFv intrabodies leads to either ER retention (sFvTacKDEL) or ER degradation (sFvTac) of IL-2Rα.

targeted to the lumen of the ER. When the anti-erbB-2 sFv intrabodies were expressed in erbB-2 transformed NIH/3T3 cells that express an oncogenically activated form of the receptor with constitutive and ligand-independent kinase activity, the sFv intrabodies bound to the receptor and prevented its transit through the ER. This resulted in the functional and stable inactivation of the oncoprotein as demonstrated by a decrease in the intensity of phosphotyrosine-containing proteins in these cells and reversion of the transformed phenotype. It should be noted that anti-erbB-2 sFv

intrabodies that did not contain the KDEL retention signal and were secreted, had less inhibitory activity than their KDEL-containing counterparts. In related studies, Deshane et al. [43] reported on the biological effects of anti-erbB-2 sFv intrabodies that bind to the extracellular domain of erbB-2 and that were targeted to the lumen of the ER, but did not contain the KDEL retention signal. Using the erbB-2 overexpressing ovarian carcinoma cell line SKOV3, these investigators demonstrated that transfection of these ER-directed (but not necessarily ER-retained) anti-erbB-2 sFvs resulted in inhibition of erbB-2 surface expression and cellular proliferation and a marked reduction in survival of neoplastic clones.

Expression of sFv intrabodies against the $p21^{ras}$ proto-oncoprotein has been reported [37,44]. In the studies by Biocca et al. [37,38], microinjection of mRNA encoding an anti-*ras* sFv intrabody into the cytoplasm of *Xenopus* oocytes was used to test the feasibility of perturbing the function of the cellular $p21^{ras}$ protein. *Xenopus* oocytes could be induced to mature meiotically in vitro by incubation with insulin, and $p21^{ras}$ had been shown to be involved in this process [157]. Oocytes microinjected with mRNA encoding for the cytoplasmically expressed anti-*ras* sFv intrabodies demonstrated an inhibition of the insulin-induced meiotic maturation [37,38]. Interestingly, oocytes injected with mRNA encoding cytoplasmic expressed complete heavy and light chains demonstrated only inhibition of histone H1 kinase activity leading the authors to conclude that interference by the whole antibody was not sufficient to block meiotic maturation [38]. Werge et al. [44] performed transient cotransfection studies in Jurkat T cells with an anti-*ras* sFv intrabody expressor and a CAT reporter gene under the transcriptional control of several copies of the binding site for the transcription factor NF-AT. These investigators demonstrated that the anti-*ras* sFv intrabody (Y13-259) interfered with NF-AT activation upon direct activation of the T cell antigen receptor, whereas activation by direct protein kinase C stimulation was less sensitive to the anti-*ras* sFv intrabody.

7. Future directions

Our results and the recent work of other groups illustrate that intrabodies provide a simple and powerful new approach to altering the in vivo function of selected microbial and cellular target proteins. As a research tool, intrabodies can be directed to individual proteins of an infectious agent or cell to identify their important functions in vivo, and offers an alternative approach to classic mutagenesis to identify functionally important regions of a microbial or cellular protein. Intrabodies should also be useful in studying complex receptor-ligand systems and signal transduction pathways, providing an attractive alternative to chemical inhibitors, dominant-negative mutants, or RNA-based strategies. Secretion of molecules such as hormones, cytokines, angiogenic molecules and inflammatory mediators should be inhibitable with ER-targeted intrabodies. Within the ER, viral glycoproteins as well as surface molecules such as cellular growth hormone receptors, surface adhesion molecules, major histocompatibility complex (MHC) class I or II molecules, etc., can be readily targeted and 'phenotypic knockouts' can be achieved. It should be possible to retain individual members of a receptor family in the ER and analyze effects of ligands on the altered cells. For example, retention of the γ_c chain could be used to explore its association with other cytokine receptors [158,159]. In our laboratory, $> 2 \times 10^5$ molecules have been removed from the cell surface (unpublished observations), a level which suggests that even highly abundant proteins can be effectively downregulated. As such, this technology may complement traditional 'gene knock-out' studies in transgenic animals [4], and may have the additional ability to 'knock-out' more than one protein target simultaneously if a conserved epitope (or structural motif) is expressed on more than one protein. Further insight into the mechanism(s) by which cellular oncoproteins alter cell growth and promote neoplastic transformation can be expected to be made through this technology [42,43].

Expression of intrabodies in other subcellular compartments such as the cytoplasm should allow the interference with different classes of proteins such as those that are involved in complex signal transduction pathways. The development of intrabodies against the cytoplasmic domains of cell surface receptors/molecules and amplified and/or

overexpressed oncoproteins may provide additional insight into their mechanism(s) of normal (or altered) signal transduction, particularly if the intrabodies are directed against specific important structural motifs in the cytoplasmic domain [160–162] or to activated forms of the receptors, i.e., phosphorylation sites [163–165]. The addition of structural motifs that will target the intrabodies to the appropriate subcellular location (i.e., myristolation signals) may further improve their efficacy [20,166]. Moreover, the addition of different functional protein domains from cytoplasmic or nuclear cellular proteins (i.e., SH2, SH3, rel homology domains) [164,167–169] will add a new dimension to this technology and may allow the construction of semi-synthetic signal transduction pathways, which could result in either inhibition or stimulation of a cellular function.

In the nucleus, complex protein interactions occur with other proteins as well as with RNA and DNA. The antibody engineering technology should already exist to create intrabodies against specific RNA and DNA structures as such antibodies occur naturally in several disease states [170–172]. This approach may allow us to directly alter the transcriptional regulation of the cell, an approach that has been reported with other protein-based strategies [173–176].

For their clinical use in a gene therapy setting, certain basic questions must be answered. First, perhaps the most important is whether transduced cells that express intrabodies will present intrabody fragments in the context of MHC class I molecules and elicit cytotoxic T cell responses. This could be beneficial if tumor cells are transduced but detrimental if normal cells (i.e., T cells) are transduced. Will human intrabodies be required or can murine intrabodies be used in this clinical setting? Although it may appear obvious that this will present a real problem to their clinical use, cells transduced with the prokaryotic neomycin (Neo/R/) gene have remained in circulation in cancer patients for over 18 months, and foreign gene expression has been demonstrated following their recovery and ex vivo characterization [177]. Second, will intrabody transduced cells have their normal in vivo cellular functions adversely altered. In a number of experiments that we have reported to date with intrabody transduced T cell lines, morphology, surface phenotype, replication rate and response to mitogenic stimulation have been normal. Third, can intrabodies be expressed at high enough levels and for long periods of time to achieve the desired clinical benefit. This latter problem may be a more general problem for transgenes which can be silenced by a number of mechanisms including deletion and methylation of DNA [178–180], than it is a specific problem for intrabodies. In this regard, further improvements in gene transfer technology may be needed. Finally, in the many examples of intrabodies that have been described, inhibition of viral and cellular protein function occurred through stoichiometric binding to their target proteins, whereas their potential biological activity could be greatly improved if catalytic functions were possible. Indeed, perhaps the real future for catalytic antibodies [181–183] lies in their use as catalytic intrabodies.

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