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Functional inhibition of transitory proteins by intrabody-mediated retention in the endoplasmatic reticulum

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ABSTRACT

Intrabodies are recombinantly expressed intracellular antibody fragments that can be used to specifically bind and inhibit the function of cellular proteins of interest. Intrabodies can be targeted to various cell compartments by attaching an appropriate localization peptide sequence to them. An efficient strategy with a high success rate is to anchor intrabodies in the endoplasmatic reticulum where they can inhibit transitory target proteins by binding and preventing them to reach their site of action. Intrabodies can be assembled from antibody gene fragments from various sources into dedicated expression vectors. Conventionally, antibody cDNA sequences are derived from selected hybridoma cell clones that express antibodies with the desired specificity. Alternatively, appropriate clones can be isolated by affinity selection from an antibody *in vitro* display library. Here an evaluation of endoplasmatic reticulum targeted intrabodies with respect to other knockdown approaches is given and the characteristics of various intrabody expression vectors are discussed. A step by step protocol is provided that was repeatedly used to construct intrabodies derived from diverse antibody isotypes producing hybridoma cell clones. The inactivation of the cell surface receptor neural cell adhesion molecule (NCAM) by a highly efficacious novel endoplasmatic reticulum-anchored intrabody is demonstrated.

1. Introduction

To study protein function or interactions in mammalian cells a wide variety of methods have been devised to inhibit gene expression or to inactivate proteins. Below a brief synopsis is given of the salient features of the most popular approaches with respect to the potential of intrabodies. In addition, possible sources of antigen-binding fragments and the choice of gene transfer vectors for intrabody expression are discussed and a protocol for ER-anchored intrabody construction from hybridoma cells is provided.

1.1. Availability, ease of use and possible side effects of small molecule inhibitors

As a result of ongoing screening efforts small molecule inhibitors for a multitude of proteins have been identified. Usually the compound can simply be added to the cell culture medium and the effects are rapid. Due to the ease of application this may be the method of choice to investigate protein function. However, for most proteins such inhibitors are not yet available and the success rate of new discoveries is declining. In addition, a critical point is the difficulty to experimentally confirm their specificity. Depending on the concentration used such compounds may have multiple known or unknown off target effects in a given experimental setup [1–3].

1.2. Gene knockout in stem cell-like cells by homologous recombination

Targeted gene knockouts inactivate a gene based on homologous recombination of the ends of a linear *in vitro* generated DNA knockout construct and chromosomal sequences. To ensure the complete loss of function with minimal side effects the entire protein coding sequence should be deleted. The deleted sequence is conventionally replaced by sequences encoding a drug resistance selection marker or by a reporter gene to facilitate the isolation of the desired cells or to monitor the promoter activity, respectively. Ongoing efforts aim at the establishment of gene knockout mouse strains for every gene in the genome. The recombinant stem cells or tissue from the resulting transgenic animals could serve as a source of cells to generate tissue-specific differentiated recombinant cell lines [4,5]. On the other hand, the augmented non-specific recombination activity in most established cell lines leads to an overwhelming background of insertions at apparently random sites in the genome. The sequence-specific recombination reliably

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works only in embryonic stem cell related cells. Therefore, these strategies are most suited for the generation of gene knockouts in stem cells or transgenic animals rather than in established mammalian cell lines. A more recent promising development involves the use of designer restriction endonucleases to stimulate site specific recombination even in established cell lines [6,7].

1.3. Accumulation of multiple mutations by random mutagenesis of whole cells

Random mutagenesis of whole cells is generally applicable for specific genes only if solely one copy of the gene exists in the genome or if the anticipated mutant proteins act in a dominant way over the function of the wild-type protein. Tools like transposon mutagenesis may be used in whole cells to generate random insertions. However, the isolation of cell clones with the desired mutation usually requires either a selection procedure or, alternatively, extensive screening. Examples where positive selection can be used include mutations in cell proliferation control genes, such as p53, p16ink, p19 or p27. Random mutagenesis will inevitably result in inadvertent mutations at other locations in the genome. Even if the desired mutant can be obtained, it may be difficult to exclude that the presence of additional uncharacterized mutations elsewhere in the genome does not interfere with the relevant phenotype. Due to the duplicated set of chromosomes in the mammalian genome and the occurrence of gene families with multiple related genes, this approach is not generally applicable [7].

1.4. Varying efficacy of antisense RNA technologies

Antisense RNA can be produced by placing cloned cDNA in reverse orientation behind a promoter. The resulting antisense RNA has been proposed to inhibit translation of the target mRNA by binding to its complementary messenger RNA and thereby forming a double stranded RNA structure [8]. However, other mechanisms leading to target RNA degradation or transcriptional silencing of the respective gene could also play a role in mammalian cells. In principle each antisense RNA could at best inactivate one mRNA molecule. In addition, the resulting dsRNA can be recognized by various intracellular receptors like TLR3 or Mda-5 and RIG-I, triggering off-target responses such as the immune-modulatory and antiviral-acting interferon system [9]. Additional side effects can be induced by dsRNA-mediated activation of enzymes like the kinase PKR that results in a general inhibition of translation, the 2′–5′ oligo(A) synthetase that is an activator of the ribonuclease RNase L, or the RNA modifying enzyme adenosine deaminase acting on RNA (ADAR) [10]. Therefore, more recent short interfering (si)RNA technologies have almost completely overcome the antisense technique.

siRNAs are natural or synthetic double-stranded (ds) RNAs, cleaved from a cellular precursor RNA or transfected into cells, respectively. siRNA length is in the range of 21–29 nucleotides. One of the two strands associates with cellular proteins to form an "RNA-induced silencing complex" (RISC). This complex mediates the binding to and silencing of complementary target mRNA sequences by inducing its degradation or possibly by translational inhibition or transcriptional silencing [11]. Since there is no reliable tool to predict which sequences are efficacious, usually several synthetic RNA sequences that are complementary to different parts of the target mRNA have to be tested. The effect is variable, depending on multiple factors such as siRNA sequence, the length of the double stranded region and on the presence of overhangs of two nucleotides at both 3′ ends of the siRNA [12]. The efficacy can be improved by using RNA analogs that are more stable, such as locked nucleic acids or morpholinos [13,14]. In comparison to full length antisense RNA less off-target effects have been reported with siRNA, presumably due to the small size of the double stranded regions it may not activate some of the dsRNA-recognizing immune receptors. On the other hand, there are reports that small interfering (si)RNAs can, albeit less efficiently, still activate some immune receptors. Even the transfection procedure itself can impose stress on the cells and may affect cellular gene expression [12,15]. Generally, a transient reduction of target gene expression can be achieved with siRNA but only rarely a complete blockage thereof.

A further development was a technique to allow stable expression of siRNAs in the target cells by introducing inverted repeats into a transcription unit of an appropriate expression vector. These repeats then form short hairpin RNA (shRNA) structures that are further trimmed to siRNA by the cellular RNA processing enzyme Dicer. The constitutive or regulated expression allows for a prolonged or reversible silencing activity independent of the time point of transfection. Appropriate naked DNA expression vectors or viral constructs can be obtained commercially, for a limited number of genes even with pretested shRNA inserts. RNA antisense techniques can be used to improve producer strains [16,17]. Furthermore, there are prospects to use these tools for therapeutic approaches [18]. For these reasons, this method has become most popular for knock-down experiments.

1.5. Efficient functional inhibition of ER-transitory proteins by ER-anchored intrabodies

It has first been shown in bacteria that the binding specificity of antibodies can be maintained in an intracellular expressed construct termed single-chain variable fragment (scFv) intrabody that solely consists of the variable domains of the heavy and light chains joined by a peptide linker [19,20]. Alternatively, some studies have included the adjacent constant antibody domains and expressed the light chain and heavy chain antibody fragments as so called Fab intrabodies which may aid to conserve the native conformation and binding specificity (Fig. 1a) [21–23]. More unusual formats include bispecific antibodies that can complex with two different proteins, and camelid single domain antibodies (nanobodies) [24–27].

Intrabodies can bind and inhibit the function of cognate proteins and for specific applications intrabodies can be viewed as an alternative to RNA-based knock-down techniques in cases where these approaches fail (Table 1) [28–30]. The advantages of intrabodies are their reliable and efficient action even in cell types such as primary macrophages that are notoriously difficult to transfect with siRNA. Furthermore, intrabodies have an excellent target binding specificity. Functional inhibition can be accomplished either by blocking the active site or by preventing crucial interactions of the target protein, for example by preventing the transport of the cognate antigen to the cell compartment where it normally fulfills its functions. One of the main advantages of using antibodies for targeting of specific molecules is that they can recognize post-translational modifications, a unique feature with respect to the other knockdown strategies. Intrabodies can be targeted to different mammalian cell compartments [31]. Intrabodies can be targeted to different intracellular compartments and in the absence of the secretory leader they are delivered to the cytoplasm. Specific targeting to the nucleus, mitochondria, or endoplasmatic reticulum (ER) can be achieved by fusing a specific localization signal sequence to the N-terminus of the intrabody [32].

Cytoplasmatic expressed intrabodies frequently do not fold correctly which has been attributed to the reducing environment that prevents formation of disulfide bonds [33]. To overcome these problems intracellular stable antibody frameworks were selected [34,35]. Alternative approaches were the use of the bacterial