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Antigen-independent selection of stable intracellular single-chain antibodies

Adrian Auf der Maur, Dominik Escher, Alcide Barberis*

ESBATech AG, Winterthurerstr. 190, CH-8057 Zurich, Switzerland

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Abstract The intracellular expression of single-chain Fv antibody fragments (scFv) in eukaryotic cells has an enormous potential in functional genomics and therapeutics [Marasco (1997) Gene Ther. 4, 11–15; Richardson and Marasco (1995) Trends Biotechnol. 13, 306–310]. However, the application of these so-called intrabodies is currently limited by their unpredictable behavior under the reducing conditions encountered inside eukaryotic cells, which can affect their stability and solubility properties [Wörn et al. (2000) J. Biol. Chem. 275, 2795–2803; Biocca et al. (1995) Bio/Technology 13, 1110–1115]. We present a novel system that enables selection of stable and soluble intrabody frameworks in vivo without the requirement or knowledge of antigens. This system is based on the expression of single-chain antibodies fused to a selectable marker that can control gene expression and cell growth. Our results show that the activity of a selectable marker fused to well characterized scFvs [Wörn et al. (2000) J. Biol. Chem. 275, 2795–2803] correlates with the solubility and stability of the scFv moieties. This method provides a unique tool to identify stable and soluble scFv frameworks, which subsequently serve as acceptor backbones to construct intrabody complementarity determining region libraries by randomization of hypervariable loops.

Key words: Intrabody; Single-chain Fv antibody fragment; Complementarity determining region; Functional genomics; Gene therapy; *Saccharomyces cerevisiae*

1. Introduction

Antibodies are highly specialized proteins with the ability to specifically bind a wide array of molecules. They are normally secreted into the extracellular environment or remain membrane-bound on the cell surface to serve as antigen receptors. Recent progress in recombinant antibody technology has turned the antibodies into important tools for many applications in biotechnology and medicine. A favorite antibody form in these fields is the recombinant single-chain Fv antibody fragment (scFv), which is expressed from a single cDNA and is composed of an antibody variable light-chain tethered to a variable heavy-chain sequence by a flexible linker [5,6]. Each variable domain contains three hypervariable loops known as complementarity determining regions (CDRs) that are embedded in the framework sequence and contribute the antigen-binding pocket. This minimal antibody form generally binds an antigen with the same specificity and affinity as the full-size parental antibody. In addition to being secreted, the scFv form can also be expressed in the cytoplasm and targeted to any compartment of any eukaryotic cell by omitting or changing the specific signal sequence [1,2]. Intracellularly expressed scFvs are called intrabodies. The potential of intrabodies is becoming increasingly recognized, in particular in the field of functional genomics, where they can be used as powerful tools to specifically target intracellular proteins, thus enabling the analysis of protein function in vivo. Intrabodies could modulate molecular events inside the cell by, for example, directly blocking a protein activity or by interfering with protein–protein interactions (see [7–11] for representative examples).

Despite this exciting perspective and a few examples of successful applications, the efficient cytoplasmic expression of scFvs is generally limited by their instability and insolubility. The reducing environment of the cytoplasm prevents the formation of the conserved intrachain disulfide bridges, thus rendering a high percentage of scFvs unstable and, as a consequence, non-functional inside the cell [4,12]. Stability and solubility of single-chain antibodies in a reducing environment represent therefore a major problem for the application of intrabodies as potential modulators of protein function in vivo. One approach to solve this problem can be provided by constructing randomized CDR libraries on scFv frameworks that have been selected for high stability and solubility in an intracellular environment.

The system presented here, named 'Quality Control', allows the selection of stable and soluble intrabodies in yeast independently of their antigen-binding specificity. We show that the activity of a selectable marker protein fused to an scFv strictly correlates with the stability of the scFv moiety. Since the selectable marker used in our assay can control cell growth by activating transcription of a conditionally essential gene, yeast colony formation on a plate, or growth in liquid culture, depends on the presence of a stable and soluble scFv antibody fragment inside the cell. Here, we validate our 'Quality Control' system by testing various anti-GCN4 scFvs that have previously been characterized for stability, solubility and their in vivo performance [3]. We show that only stable and soluble scFv moieties permit activation of the specific reporter genes. This approach allows efficient screens of scFv libraries to isolate 'super-stable' frameworks suitable for intracellular applications.

*Corresponding author. Fax: (41)-1-635 6811.
E-mail address: barberis@esbatech.com (A. Barberis).

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2. Materials and methods

2.1. Plasmids and yeast strains

The plasmids expressing the various anti-GCN4 scFv fusion proteins were constructed as follows. The Gal4 activation domain (AD) (amino acids 768–881) was amplified by polymerase chain reaction (PCR) using pGAD424 (Clontech) as template. Both primers (upstream primer: 5′-CCA TGG GCC CAA GCT TTG CAA AGA TGG ATA AAG-3′; downstream primer: 5′-TTT GGG CCC GAA GAA CCG CCA CCA CCA GAA CCG CCT CCA CCA GAG CCA CCA CCA CCA GGC CTG ATC TCT TTT TTT GGG TTT GGT G-3′) contain an *Apa*I site suitable for cloning the Gal4 AD including the SV40 T-antigen nuclear localization signal N-terminal to the different scFv variants in the context of pESBA-Act [3]. The AD and the single-chain antibodies are seperated by a $(GGGS)_3$ linker encoded by the downstream primer. The DNA fragments encoding amino acids 263–352 of Gal11 or Gal11P were amplified by PCR with the primer pair 5′-CAT GCC ATG GTT CCT CAA CAG CAG CAA ATG CAA C-3′ and 5′-CAT GCC ATG GCG CTA GCC AAA GCT TGG ATT TTT CTC AGG-3′. The constructs pSO23 and pSO32 [13] were used as templates for the Gal11 wild-type fragment or the Gal11P sequence, respectively. Both primers are flanked by an *Nco*I site in order to clone the digested PCR product upstream of the Gal4 AD. All clones were sequenced to confirm in-frame fusion and correct orientation of the inserts. The mammalian expression vectors encoding the various Gal4 (1–147)-VP16-scFv fusion proteins are based on a plasmid expressing Gal4 (1–147) under the control of the CMV promoter. The VP16 AD was amplified by PCR with the primers 5′-ATC GGA TCC CAG CCC CCC CGA CCG ATG TCA GCC TG-3′ and 5′-TTC CGT CGA CCG GAT ATC GAA CCA CCA CCA CCG TAC TCG TCA ATT CCA AG-3′ and cloned via *Bam*HI and *Sal*I downstream of Gal4 (1–147). All scFv variants cloned in pESBA-Act [3] were first digested with *Nco*I and subsequently treated with Klenow in order to obtain blunt ends. The same DNA was then digested with *Sal*I and the released scFv inserts (*Nco*I [blunt ended]–*Sal*I) were cloned downstream of VP16 of the *Eco*RV–*Sal*I-digested Gal4 (1–147)-VP16 vector. The yeast expression vectors encoding the LexA-AD-scFv fusions were obtained by cloning a LexA fragment and the respective scFv fragment fused to the AD of Gal4 into pAB125 digested with *Hin*dIII and *Sal*I. The LexA moiety was released by *Hin*dIII–*Sma*I digestion of plex113 and the AD-scFv fragment (*Nco*I [blunt ended]–*Sal*I) was obtained from a plasmid expressing the AD-scFv fusion (see above). The yeast-integrating reporter plasmid pDE103, which carries the divergently oriented LacZ and *HIS3* genes under the control of the natural UAS_G from the GAL1–GAL10 regulatory sequences, was linearized at the *Afl*II site in the HIS3 3′ untranslated region (UTR) and integrated into the his3Δ200 locus of JPY9 [14] resulting in YDE172 (*MATα ura3-52 leu2 Δ 1 trp1d63 lys2 Δ 385 gal4Δ 11*). The integrating reporter plasmid pDE200 [14] drives the expression of LacZ and HIS3 from a bidirectional promoter with six LexA-binding sites. This plasmid was linearized at the *Afl*II site in the HIS3 3′ UTR and integrated into the his3Δ200 locus of JPY9 resulting in YDE173.

2.2. β-Galactosidase assays

Gene expression in yeast was monitored by using liquid β-galactosidase assays as previously described [3]. All assays were conducted with duplicate or triplicate samples and were repeated at least once. Normalized β-galactosidase activity obtained with the λ-graft fusion proteins was generally set to 100% in all experiments.

2.3. Western blot analysis of scFv fusion proteins

5 ml cultures were grown to an optical density (OD_{600}) of 2. One half of the culture was used for the preparation of whole cell extracts as described [15]. The other half was subjected to a gentle isolation of soluble proteins with Y-PER™ (a Yeast Protein Extraction Reagent from Pierce) as described previously [3]. Samples of soluble and whole cell extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes, following standard protocols. An antibody from Santa Cruz Biotechnology, Inc. specific for the AD of Gal4 (Gal4AD (sc-1663)) was used to detect the Gal11P-Gal4AD-scFv fusion proteins.

2.4. Mammalian cell cultures, transfection and luciferase assays

HeGlu cells [16] were cultured in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin and 2 mM L-glutamine. Transient transfections were carried out by the calcium phosphate method [17] in 100-mm tissue culture plates with 5 μg of a CMV promoter driven Gal4 (1–147)-VP16 scFv expression plasmid and 1 μg of a LacZ expression vector as reference for transfection efficiency. The following day, cells were washed and 48 h after transfection they were harvested. Cells were resuspended in 100 μl extraction buffer (100 mM potassium phosphate pH 7.8, 1 mM dithiothreitol) and lysed by freezing and thawing three times. Supernatants were clarified by centrifugation (5 min, 13 000 rpm), 10 μl of extract was mixed with 100 μl luciferase assay solution (Promega) and analyzed in a luminometer (EG&G Berthold Lumat LB 9507). β-Galactosidase assay was performed according to standard methods using 50 μl of the extract and luciferase units were normalized according to β-galactosidase values. All measurements were performed from two independent transfections. Luciferase activity obtained with the λ-graft fusion protein was arbitrarily set to 100%.

3. Results and discussion

The technique presented here to identify intracellularly stable and soluble scFv frameworks is based on the assumption

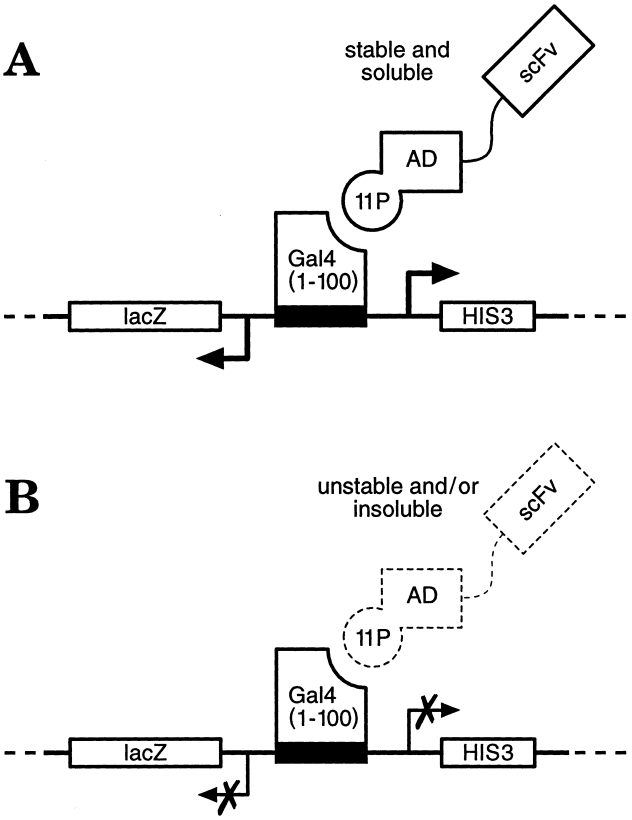

Fig. 1. Schematic representation of the 'Quality Control' system to select stable and soluble scFv antibody fragments independently of their antigen-binding specificity. An scFv is fused to a selectable marker protein comprising a transcriptional AD and a peptide derived from Gal11P, which can mediate the specific interaction with the DNA-bound Gal4 (1–100) fragment. The conceptual basis of the system is that the activity of the fusion protein is dependent on the stability and solubility of the scFv moiety. A: A fusion protein carrying a stable and soluble scFv is tethered to DNA via the Gal4 (1–100)–Gal11P interaction where it activates transcription of the divergently oriented *HIS3* and *lacZ* reporter genes (bent arrows). B: Unstable and/or insoluble scFv moieties cause inactivation of the whole fusion protein (dashed line), thus resulting in the lack of expression of the reporter genes (crossed arrows).

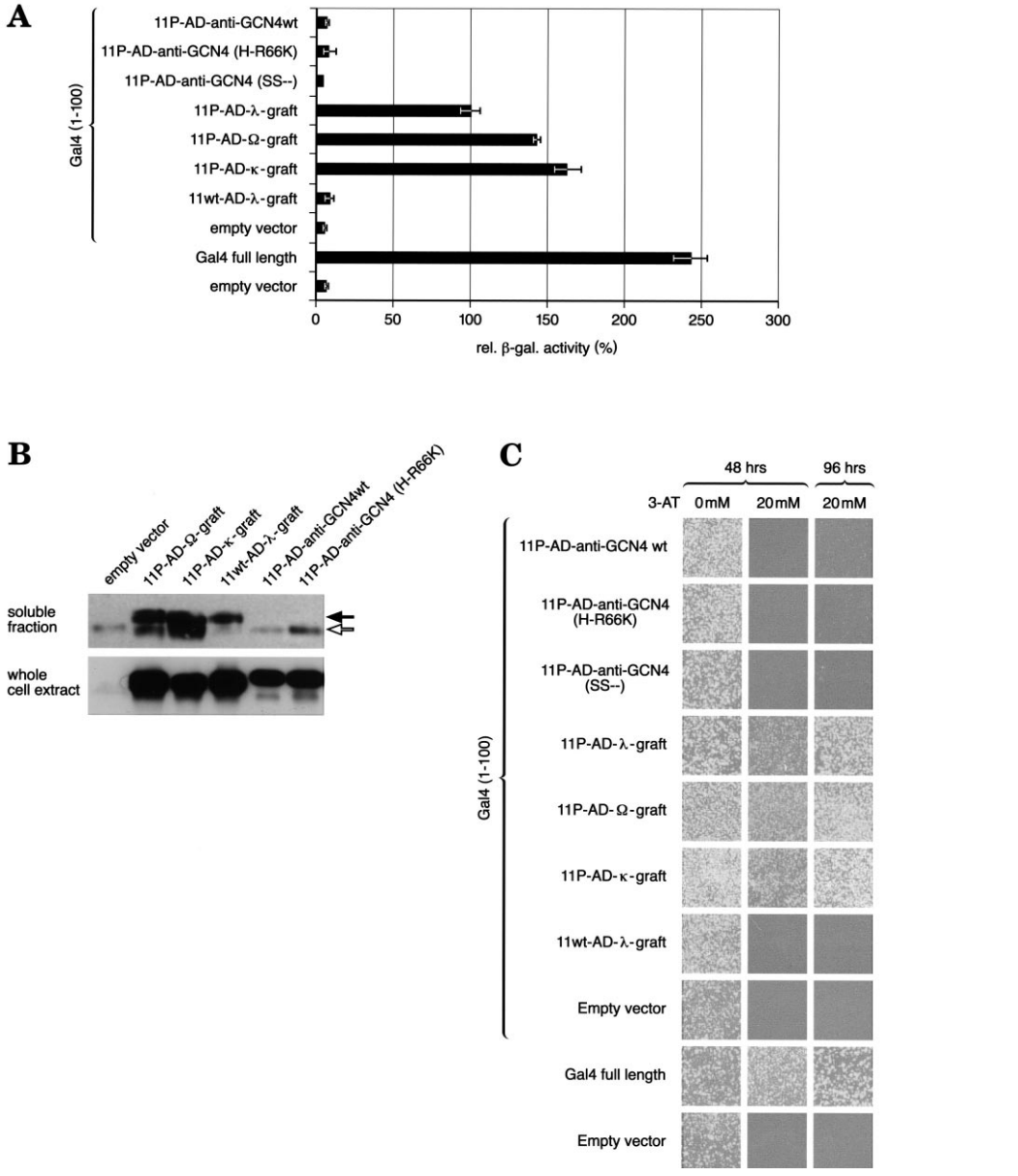

Fig. 2. Reporter gene activation is dependent on the stability and solubility of various anti-GCN4 scFv variants. A: β-Galactosidase assay to measure activation of the *lacZ* reporter gene. The reporter yeast strain was transformed with plasmids expressing one of the indicated scFv hybrid proteins together with the Gal4 (1–100) fragment. Gal4 full-length and an empty vector were used as a positive and a negative control, respectively. A hybrid protein carrying a peptide derived from the wild-type Gal11 (11wt-AD-λ-graft), which is unable to interact with Gal4 (1–100), was also used as a control. The soluble and highly stable Ω-, λ-, and κ-graft scFv variants enabled the respective 11P-AD fusion proteins to strongly activate the *lacZ* reporter gene, while the less stable scFv variants (anti-GCN4wt, anti-GCN4H-R66K, anti-GCN4SS--) did not allow significant reporter gene activation. Relative β-galactosidase activity (rel. β-gal activity) elicited by the 11P-AD-λ-graft fusion protein was arbitrarily set to 100%. B: Western blot analysis showing the correlation between solubility of the scFv fusion proteins and activation of the *lacZ* reporter gene. The fusion proteins (11P-AD-scFv) were detected with an antibody specific for the AD of Gal4. The open arrow indicates a background band present in all lanes including the 'empty vector' control not expressing any fusion protein. Only the graft variants (11P-AD-Ω-graft, 11P-AD-κ-graft, 11P-AD-λ-graft) were detected in the soluble fraction (indicated with a black arrow) while all scFv fusion proteins were very abundant in whole cell extracts. C: Growth on selective plates containing 3-AT correlates with the gene activation potential of the tested scFv fusion proteins. Yeast cells transformed with the same set of plasmids as in A were spread on selective plates lacking histidine and supplemented with 0 mM or 20 mM 3-AT solutions. Cell growth was monitored after 48 and 96 h incubation at 30°C.

that the stability and the solubility (and therefore the activity) of a fusion protein composed of a constant, selectable marker fused to an scFv are determined by the quality of the scFv moiety. To test this hypothesis, we fused a number of well characterized scFvs to a marker protein that is composed of a peptide linked to the transcriptional activation domain (AD) of Gal4 (Fig. 1). The peptide was derived from the mutant form of the yeast protein Gal11 called Gal11P. It has previously been shown that only the mutant Gal11P peptide, but not the wild-type sequence, specifically interacts with the dimerization region of the DNA-binding Gal4 (1–100) fragment [18]. The AD of Gal4 is known to activate transcription when tethered to DNA [19]. According to the hypothesis outlined above, a fusion protein bearing a soluble and stable scFv

should be tethered to DNA via the Gal4 (1–100)–Gal11P interaction and, thus, activate transcription of the reporter genes.

Several defined scFv fragments displaying very similar antigen-binding properties, but different in vitro stability and in vivo performance [3], were fused to the Gal11P-Gal4AD marker protein. These scFv variants bind specifically the so-called leucine zipper of the yeast transcription factor Gcn4. The original anti-GCN4 wild-type (anti-GCN4wt) scFv was obtained by ribosome display from a library constructed from an immunized mouse [20]. Several different framework variants of this anti-GCN4 scFv have been constructed and described in our recent work [3]. These variants have been extensively characterized for their in vitro stability and solubility, as well as for their in vivo performance as inhibitors of the Gcn4 transcriptional activity in yeast [3]. These scFv fusion proteins and the DNA-binding Gal4 (1–100) fragment were co-expressed in a yeast strain (YDE172) containing the reporter genes *HIS3* and *lacZ* under the control of four Gal4-binding sites (Fig. 1). Yeast cells expressing these different proteins were grown in culture and subjected to a β-galactosidase assay to quantify expression of the *lacZ* reporter gene. Cells expressing full-length Gal4 or carrying an empty vector were used as positive and negative controls, respectively. A fusion protein carrying a Gal11 wild-type peptide, which does not interact with Gal4 (1–100), was used as an additional control. Fig. 2A shows that the Gal11P fusion proteins bearing the stable scFv moieties called λ-graft and κ-graft [3] were able to strongly activate *lacZ* expression, while those harboring the unstable scFvs (anti-GCN4wt, anti-GCN4H-R66K, anti-GCN4SS-- [3]) were unable to stimulate *lacZ* expression. The so-called Ω-graft, which differs from the λ-graft by only three amino acids in the framework sequence, displayed an approximately 40% better in vivo performance than the λ-graft (Fig. 2A), which is similar to the performance observed in antigen-dependent in vivo interaction experiments (our unpublished results).

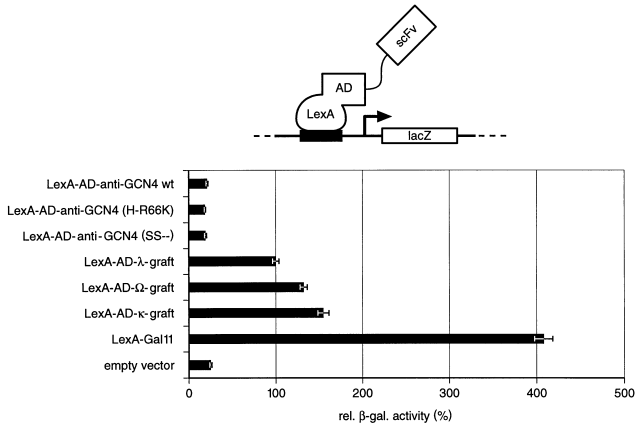

Fig. 3. Stability and solubility of various anti-GCN4 scFv framework variants determine the activity of different types of selectable marker proteins. A yeast strain carrying the *lacZ* reporter gene outlined at the top of the panel was transformed with plasmids expressing the indicated anti-GCN4 scFv variants fused to a transcriptional activator comprising the DNA-binding LexA and the AD of Gal4. The strong activator LexA-Gal11 [18] and an empty vector were used as positive and negative controls, respectively. Relative β-galactosidase activity (rel. β-gal activity) elicited by the LexA-AD-λ-graft fusion protein was arbitrarily set to 100%.

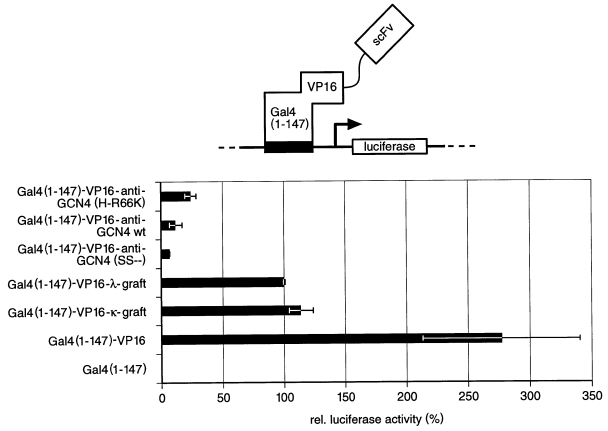

Fig. 4. Stability and solubility of various anti-GCN4 scFv framework variants determine the activity of a transcriptional activator in mammalian cells. HeLa cells carrying the integrated luciferase reporter gene depicted at the top of the panel were transfected with plasmids expressing the indicated anti-GCN4 scFv variants fused to the transcriptional activator Gal4 (1–147)-VP16. Gal4 (1–147)-VP16 and Gal4 (1–147) alone were used as positive and negative controls, respectively. Relative luciferase activity (rel. luciferase activity) elicited by the Gal4 (1–147)-VP16-λ-graft fusion protein was arbitrarily set to 100%.

The solubility of the different Gal11P-Gal4AD-scFv fusion proteins in yeast was assessed by Western blot analysis. Two different protocols were applied for the preparation of yeast cell extracts (see Section 2). One protocol facilitates isolation of soluble proteins, while the other protocol is commonly used to prepare whole cell extracts. Significant amounts of proteins were detected in the soluble fraction in the case of the Ω-, κ-, and λ-graft variants fused to Gal11P-Gal4AD (Fig. 2B). The other fusion proteins comprising the unstable scFvs (anti-GCN4wt and anti-GCN4H-R66K [3]) appeared to be completely insoluble since they were not detectable in the fraction containing soluble proteins. Nevertheless, all Gal11P-Gal4-AD-scFv fusion proteins were very abundant and easily detectable in whole cell extracts. The observed differences in solubility between the scFv fusion proteins were highly reproducible and correlated with results of previous experiments in which solubility was assessed for the non-fusion scFvs [3].

These results, taken together with the biochemical characterization of the various scFvs [3], show a correlation between reporter gene activation (β-galactosidase activity) by the fusion protein and the in vitro stability and solubility of the single-chain moiety. The Gcn4 antigen-binding activities of the scFv antibody fragments in the context of these fusion proteins were tested by comparing their ability to inhibit Gcn4 function in yeast with that of the originally characterized scFvs [3]. All scFv hybrid proteins tested here reduced Gcn4-dependent gene expression to the same extend as the original, non-fusion scFvs (data not shown). Thus, the fusion of a marker protein did not alter the biological activity of the scFv antibody fragments.

The reporter strain used in this study (YDE172) also contains the *HIS3* gene under the control of four Gal4-binding sites (Fig. 1). Activation of this reporter gene allows growth selection on plates lacking histidine and containing 3-aminotriazole (3-AT), which is a competitive inhibitor of the *HIS3* gene product. The same yeast cells that were subjected to the liquid β-galactosidase assay (Fig. 2A) were spread on selective

plates lacking histidine and containing different concentrations of 3-AT, as well as on non-selective control plates. Under non-selective conditions, no difference in the growth rate could be observed between yeast cells harboring the various scFv fusion variants. Under selective conditions, a 20 mM 3-AT concentration was already sufficient to suppress growth of yeast cells carrying an empty vector or expressing a fusion protein with unstable and insoluble scFv moieties (Fig. 2C). Even after the observation period of 4 days, yeast cells expressing unstable fusion variants were unable to grow in the presence of 3-AT concentrations ranging from 10 mM to 60 mM (Fig. 2C and data not shown). In contrast, stable and soluble scFv moieties such as the λ-graft, the κ-graft, and the Ω-graft endowed the respective fusion proteins with the ability to stimulate growth of the yeast cells under these selective conditions (Fig. 2C). These results indicate that this growth selection procedure allows to distinguish intracellularly stable scFvs from unstable ones.

We then tested whether the correlation between the in vivo activity of the Gal11P-Gal4AD selectable marker and the stability of the scFv antibody fragment that was fused to it would also be observed with another fusion protein bearing a different selectable marker. For this purpose, we substituted the Gal11P peptide of the Gal11P-Gal4AD-scFv fusion proteins described above with the DNA-binding LexA protein. These fusion proteins, if stable and active, should directly and specifically bind DNA and activate transcription from a nearby promoter. The LexA fusion proteins were expressed in a yeast strain YDE173 containing a *lacZ* reporter gene under the control of six LexA-binding sites. Activation of the reporter gene was quantified by β-galactosidase assays. Fig. 3 shows that, also in this context, the stability and the solubility of the scFv antibody fragments determined the functionality of the hybrid proteins in yeast cells (compare Fig. 3 with Fig. 2A). In addition, similar results as in Fig. 2C were obtained from a growth selection assay with the LexA fusion proteins expressed in YDE173 (data not shown).

To determine whether these characterized scFvs have the same properties when expressed in mammalian cells, we adapted our 'Quality Control' system to mammalian expression vectors. We modified our system such that the scFv fragments tested in yeast (see above) were fused to a strong mammalian transcriptional activator consisting of the Gal4 DNA-binding domain and the VP16 AD (Gal4 (1–147)-VP16) [21]. HeGlu cells, which are HeLa cells carrying an integrated luciferase reporter gene under the control of Gal4-binding sites [16], were transfected with these constructs as well as with a plasmid expressing Gal4 (1–147)-VP16 alone (positive control) or with an empty vector (negative control). After 48 h of incubation, reporter gene expression was monitored by measuring luciferase activity (Fig. 4). The relative levels of activation of the luciferase reporter gene by the different scFv fusion proteins correlated with the levels of β-galactosidase activities elicited by the respective scFv fusion proteins in yeast. The λ-graft and κ-graft, which were stable and soluble in yeast and thus allowed gene activation by the respective fusion proteins, also mediated strong activation of the reporter gene in mammalian cells. The other scFv framework variants, which did not allow reporter gene activation in yeast, could also not significantly stimulate reporter gene expression in mammalian cells (compare Fig. 4 with Fig. 3). Thus, our 'Quality Control' system allows us to exploit the technical advantages of the yeast cells to identify intrabodies that are also functional in mammalian cells.

4. Conclusion

Low solubility and instability represent a major stumbling block for the efficient exploitation of scFv antibody fragments to knock out or modulate protein function inside eukaryotic cells. So far, no reliable rules are available to make predictions about antibody structures that can tolerate the reducing environment of the cytoplasm, and individual biochemical analysis of scFvs is a long and tedious procedure. Our goal was to develop a system that allows a high-throughput screen of scFv antibody fragments to identify those that are highly stable in an intracellular (reducing) environment. In order to screen a large number of diverse scFv sequences, the selection assay could not rely on antibody–antigen interactions. This is, to our knowledge, the first report on an in vivo method to identify and analyze intrabodies for stability and solubility independently of their antigen-binding properties. To this end, we have constructed fusion proteins composed of scFv sequences fused to a constant marker that provides a selectable activity in yeast by controlling expression of defined reporter genes. Our results indicate that the overall stability and the function of the fusion protein are determined by the scFv portion. We show that the degree of reporter gene activation is directly proportional to the stability and solubility of the single-chain antibody and can be quantified by measuring expression of the reporter genes. Analysis of the soluble fraction for each fusion protein revealed a strong correlation between solubility and reporter gene activation. Thus, our 'Quality Control' system is suitable to validate single-chain antibodies that can cope with intracellular conditions. With the possibility to perform growth selection assays, our system allows to rapidly and efficiently screen single-chain antibody libraries to isolate a set of 'super-stable' frameworks. Such frameworks subsequently serve as acceptor backbones to construct CDR libraries by randomization of one or more hypervariable loops. Additionally, our system can be used in an 'intrabody evolution process' to improve the in vivo performance of specific single-chain antibodies that are poorly stable, and thus only weakly functional under reducing conditions.

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