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Direct *in Vivo* Screening of Intrabody Libraries Constructed on a Highly Stable Single-chain Framework*

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Single-chain Fv antibody fragments (scFv) represent a convenient antibody format for intracellular expression in eukaryotic or prokaryotic cells. These so-called intrabodies have great potential in functional genomics as a tool to study the function of newly identified proteins *in vivo*, for example by binding-induced modulation of their activity or by blocking interactions with other proteins. However, the intracellular expression and activity of many single-chain Fvs are limited by their instability and folding efficiency in the reducing intracellular environment, where the highly conserved intrachain disulfide bonds do not form. In the present work, we used an *in vivo* selection system to isolate novel antigen-binding intrabodies. We screened two intrabody libraries carrying a randomized third hypervariable loop onto the heavy chain of a stable framework, which had been further optimized by random mutagenesis for better behavior in the selection system, and we biophysically characterized the selected variants to interpret the outcome of the selection. Our results show that single-framework intrabody libraries can be directly screened *in vivo* to rapidly select antigen-specific intrabodies.

Antibodies are pivotal components of the vertebrate immune system that can bind to almost any molecule with a high degree of specificity and affinity. These characteristics have been exploited to turn the natural antibodies into powerful biotechnological tools in diagnostic and therapeutic applications. Advances in recombinant DNA technology have facilitated the manipulation, cloning, and expression of the antibody genes in a wide variety of hosts (1–3). Several forms of antibodies have been constructed to obtain derivatives that carry the binding site in a smaller assembly. One of the minimal forms still retaining the full binding site is the single-chain Fv fragment (scFv)[1] (4–6). In the scFv form, the variable regions of the heavy and the light chains, which bear the hypervariable loops (or complementarity determining regions (CDR)), are connected by a flexible linker, allowing the expression of the protein from a single cDNA sequence.

Natural antibodies, which are secreted by plasma cells, have evolved to function in an extracellular environment. In contrast to full-length, double-chain antibodies, the scFv antibody form can in principle be readily expressed also in the cytoplasm of eukaryotic cells and directed to any compartment to target intracellular proteins and thus evoke specific biological effects. Hence, intracellular scFvs, also known as "intrabodies," might have a great potential in functional genomics by blocking or modulating the activity of a growing number of newly identified proteins, thereby contributing to the understanding of their functions. In the long run, intrabodies might even be used in therapeutic applications, possibly in gene therapy settings.

However, there are only few examples of successful applications (7–13), and the cytoplasmic expression of scFvs is generally confronted with the difficulties concerning the stability, solubility, and aggregation tendency of the intrabody. Because the reducing environment of the cytoplasm prevents the formation of the highly conserved intradomain disulfide bridges, very stable frameworks are needed that fold in the absence of the disulfide bond and do not undergo aggregation (14, 15). The sequence requirements that make an scFv very stable such that it might also function in an intracellular environment are only now emerging.[2]

We have recently described a model system to measure the binding activity of a series of cytoplasmically expressed scFvs in yeast (15). These scFvs, which carry the same CDR loops on different frameworks, were all directed against the dimerization domain of the sequence-specific transcriptional activator Gcn4 (16). Heterologous expression in yeast of some of these specific scFvs inhibited activation of transcription by Gcn4, presumably by blocking the homodimerization of this protein. With our experiments, we demonstrated a clear correlation between *in vivo* performance and *in vitro* stability of the different anti-GCN4 variants. Indeed, a framework-engineered stabilized scFv showed significantly stronger activity, thus indicating that thermodynamic stability is one of the most important parameters for intracellular activity. Consequently, improving the thermodynamic stability of scFvs might be a promising strategy to increase the yield of functional intrabodies.

In this work, we present a further development of our approach (i) to select for scFv frameworks that behave better *in vivo* and (ii) to screen random CDR libraries constructed on selected frameworks for identifying intracellular antigen-binding single-chain antibodies directly. We have used a selection assay in yeast that allows the isolation of functional intrabod-

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[1] The abbreviations used are: scFv, single-chain Fv; CDR, complementarity determining regions; AD, activation domain; 3-AT, 3-aminotriazole; wt, wild type; RIA, radioimmunoassay;

[2] S. Ewert, T. Huber, A. Honegger, and A. Plückthun, submitted for publication.

ies based on their ability to bind the cognate antigen *in vivo*. In this assay, which is an adaptation of the yeast two-hybrid system (17), the scFv is linked to a transcriptional activation domain, whereas the target antigen is linked to a DNA-binding domain. A specific interaction between the scFv fusion protein and the antigen leads to the activation of expression of a selectable reporter gene. We successfully applied this selection system in combination with random mutagenesis to test and optimize the *in vivo* performance of an scFv fragment. In addition, the system was used to identify CDR-H3 sequences binding to either the original or a new antigen *in vivo*. We also have characterized some of the resulting scFvs to be able to interpret the properties of this intracellular selection system.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—Plasmids expressing various anti-GCN4 fusion proteins were constructed as follows. The Gal4 activation domain (amino acid residues 768–881) was amplified by the 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 activation domain including the SV40 T-antigen nuclear localization signal N-terminal to the different scFvs in the context of pESBA-Act (15). The activation domain and the single-chain antibodies are separated by a $(GGGS)_3$ linker encoded by the downstream primer. All clones were sequenced to confirm in-frame fusion with the Gal4 activation domain. The plasmids expressing the LexA proteins fused to the leucine zippers of Gcn4 or human c-Jun were generated by PCR. The Gcn4 leucine zipper (Swiss-Prot accession number P03069, amino acids 247–281) was amplified by PCR from genomic DNA with the following primer pair: upstream primer, 5′-GGA ATT CCG TTC TCG TGC GAG AAA GTT-3′; downstream primer, 5′-GGA ATT CTA ATA TGG TGG AGT CAG CTG AG-3′. The resulting PCR product was digested with *Eco*RI and cloned into plex113 (18) cut with *Eco*RI. The leucine zipper of the human c-Jun protein (Swiss-Prot accession number P05412, amino acids 274–314) was generated by PCR from plasmid pAB140 [3] with the primers 5′-CGG GGA TCC ACC GCA TCG CTG CCT CCA AGT G-3′ and 5′-GCT CTA GAG CTC AAA ATG TTT GCA ACT GCT GCG TTA G-3′ carrying a *Bam*HI and *Xba*I site, respectively. The DNA fragment was digested with the appropriate enzymes and cloned into *Bam*HI- and *Xba*I-digested plex113 (18). Expression of these LexA derivatives is controlled by the alcohol dehydrogenase (*ADH*) promoter. The plasmids are of Ars/Cen type, harboring the *LEU2* selection marker. To introduce random amino acid mutations throughout the λ-graft framework (see below), mutagenic PCR was performed as described (19). The following primer pair was used to mutagenize the scFv portion by error-prone PCR: 5′-CAA CTA GTA ACT ATG CCT CCT GGG TC-3′ upstream; 5′-TGT TGG TGA CCA TAA CAC C-3′ downstream. The PCR product was digested with *Spe*I and *Sal*I and cloned into the Gal4AD-λ-graft expression vector.

Three amino acid exchanges in the heavy chain of the λ-graft framework were selected in a random mutagenesis of the λ-graft framework and were combined into a single framework, dubbed "Ω-graft": H-N84D, H-G85C, and H-K86E (for nomenclature see Ref. 20). These amino acid changes were introduced by site-directed mutagenesis and confirmed by sequencing. Randomization of three or eight amino acids in the CDR-H3 of the variable heavy chain was performed by a PCR-based method. Both libraries (3- and 8-mer) were based on the Ω-graft framework and were fused to the Gal4 activation domain. A 2-μm vector was used that carries an *ACT1* promoter and a *TRP1* selection marker.

The integrating reporter plasmid pDE200 (21) carries the *lacZ* and *HIS3* reporter genes expressed from a bi-directional promoter with six LexA-binding sites. This plasmid was linearized at the *Afl*II site in the *HIS3* 3′-untranslated region and integrated into the his3Δ200 locus of JPY9 (22) (*MATα ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385 gal4Δ11*). Correct integration was confirmed by genomic PCR and functional assays. This reporter strain was named YDE173 and was used for growth selection and to quantify reporter gene activation by measuring β-galactosidase activity. The yeast strain YAdM2xGCN4-150, which expresses the enzyme β-galactosidase under the control of two Gcn4-binding sites, is described elsewhere (15).

[3] A. Barberis, unpublished data.

Yeast transformation was performed according to the standard lithium acetate protocol. Transformants were selected by using auxotrophic markers for the corresponding plasmids.

Serial Dilution and Spotting of Yeast Cells—Transformed yeast cells were grown overnight at 30 °C in drop-out medium (−Trp/−Leu). The saturated cultures were serially diluted in water (dilution factor 5) starting with a concentration of 10^6 cells/ml. Always 10 μl of each dilution were spotted on selective plates (−Trp/−Leu/−His) containing 0, 40, or 80 mM of 3-aminotriazole. Six different dilutions of each transformant were spotted, which theoretically corresponds to 10,000, 2000, 400, 80, 16, and 3 cells per spot, respectively. The plates were incubated at 30 °C and scanned after 48, 72, and 120 h.

Liquid β-Galactosidase Assay—The β-galactosidase assay in solution was performed using permeabilized cells as described (23). Activity was normalized to the number of cells assayed. All measurements were performed in triplicate, and averaged values are given.

Plasmid Rescue Protocol—Five ml of a saturated yeast overnight culture was grown in media solely selecting for the library plasmid. Cells were centrifuged, and pellets were resuspended in 200 μl of H_2O and 200 μl of yeast lysis solution (2% Triton X-100; 1% SDS; 100 mM NaCl; 10 mM Tris-HCl, pH 8; 1 mM EDTA). The suspension was mixed with about 200-μl glass beads and 200 μl of phenol/chloroform, and the resulting mixture was vortexed. After centrifugation, the aqueous phase was subjected again to phenol/DCM and DCM extract, and finally precipitated with ethanol. The DNA was resuspended in 50 ml of Tris-EDTA buffer (TE) and treated with RNase. Electrocompetent *Escherichia coli* cells were transformed with about 0.2 μl of this DNA, and transformed cells were screened for the presence of the scFv-encoding plasmid.

Expression of Single-chain Fv Fragments—For *in vitro* analysis, the single-chain Fv fragments were expressed and isolated from the bacterial periplasm. Genes encoding the scFvs were cloned into the expression vector pIG6 (24), containing a signal sequence and a C-terminal His_6 tag sequence. The constructs were expressed for 4 h at 25 °C. The cells were harvested and disrupted by French press. The single-chain Fv fragments were purified using immobilized metal ion affinity chromatography. The elution fraction was concentrated using Centriprep concentrators (Millipore).

Equilibrium Renaturation of Single-chain Fv Fragments—The intrinsic fluorescence of the scFv fragments was detected to follow renaturation and denaturation. A 5 μM solution of the purified single-chain Fv fragments was reduced in 6 M urea in the presence of 100 mM dithiothreitol (pH 9, for 3 h at 30 °C). The reduced protein was diluted to urea concentrations of 0.6–8 M and incubated for 12 h at 4 °C. All measurements were done in 20 mM Hepes buffer (150 mM NaCl, pH 7.4) using a Shimadzu RF-5000 spectrofluorimeter. The center of spectral mass was calculated from the fluorescence spectra as described (25). The curves were normalized and analyzed for relative stabilities as described (15).

Radioimmunoassay (RIA)—The scFv portion from five scFv-fusion constructs, carrying different randomized residues in the CDR-H3 loop on the same framework (Ω-graft), namely ALF, GFA, GLF, GLV, and GLW (position 109–136 (20)), and the original clone λ-graft were amplified in three subsequent PCRs. In the first PCR, the scFv portion was amplified as follows: upstream primer, 5′-TCC ATG GCG GAC TAC AAA GAT CCG GAT ATC GTT ATG ACC CAA TCT T-3′; downstream primer, 5′-TGT TGG TGA CCA TAA CAC C-3′ annealing in the 3′-terminator sequence of the expression vector. This PCR product served as template for the second PCR using the same downstream primer and a nested upstream primer: 5′-AGA CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA TCC ATG GCG GAC TAC AAA GAT-3′, introducing a ribosome entry site. The last PCR was performed using again the same downstream primer and another nested primer: 5′-ATA CGA AAT TAA TAC GAC TCA CTA TAG GGA GAC CAC AAC GG-3′, which carries the T7 promoter (26). The last PCR product was directly used for *in vitro* transcription, and the RNA was purified by LiCl precipitation (27). Equal amounts of RNA were used for *in vitro* translation using an *E. coli* S-30 translation system (28). Translation of 10 μg of mRNA was performed for 45 min at 37 °C in a 110-μl reaction as described (26) in the presence of 0.6 μM [^{35}S]methionine. The translation mix was split into aliquots, and milk in PBST (10 mM sodium phosphate, 50 mM NaCl, 15 mM KCl, 0.05% Tween 20, pH 7.5) was added to an end concentration of 1%. To two of the three aliquots, different amounts of the peptide Gcn4(7P14P) RMKQLEPKVEELLPKNYHLENEVARLKKLVGER (26) were added as a competitor to an end concentration of $2.5 \cdot 10^{-7}$ M and $1.5 \cdot 10^{-8}$ M, respectively, and the tubes were shaken at room temperature for 2 h. The protein was bound to a standard enzyme-linked immunosorbent assay plate (Nunc) that

was first coated with 100 μl of 4 μg/μl Neutravidin overnight and 100 μl of a 290 nM solution of the biotinylated peptide Gcn4(7P14P) for 30 min and blocked for 4 h at room temperature with 4% milk in PBST. Each antibody was split and bound in duplicates to the plate for 15 min at room temperature in the presence or absence of competitor peptide. After washing 5 times with PBST, bound protein was eluted with 0.1 M triethylamine and quantified in a scintillation counter (Wallac).

Affinity Determination with Inhibition BIAcore—Affinities of single-chain Fv fragments were measured using surface plasmon resonance by the inhibition method as described previously (26) on a BIAcore 3000 instrument. Inhibition of the purified single-chain Fv fragments was performed with the peptide Gcn4(7P14P), which remains monomeric in solution. The protein was diluted to about 5 nM and equilibrated with different concentrations of the peptide Gcn4(7P14P) overnight ranging from 0 to 15 nM. All measurements were done in Hepes buffer (20 mM Hepes, 150 mM NaCl, pH 7.4) on a CM5 sensor chip (BIAcore) previously coated to maximal density with Neutravidin (Pierce) and biotinylated peptide (7P14P)Gcn4. All curves were measured in triplicate, and the averaged data were fitted as described (26) (SigmaPlot).

Structural Modeling—The structural model of the Ω-graft variant, a nickname of a triple point mutant of the λ-graft with the CDR-H3 sequence ALFDY, is based on the crystal structure of the originally selected (26) anti-GCN4 scFv in complex with the antigen[4] and the crystal structure[4] of the framework donor (29). Both domains of the λ-graft were individually superimposed with the domains of the anti-GCN4 scFv in complex with the antigen, as seen in the crystal structure. Interface residues differing between the crystal structure of the anti-GCN4 binder and λ-graft (which is, with the exception of three outer loop residues, identical to the interface of Ω-graft) were energy-minimized. The same was done with the mutated residues in the outer loop of V_H (which is different between λ-graft and Ω-graft) and with the mutated residues in CDR-H3.

RESULTS

Activation of Transcription by the Interaction between an Antigen and an Intrabody Correlates with the in Vitro Stability and in Vivo Performance of the Intrabody Moiety—In our recent work (15), we demonstrated that stability is the key component for the function of an intrabody in the reducing intracellular environment. To obtain a suitable model system, we had studied scFvs that had been first selected *in vitro* to bind antigen (26). Subsequently, a series of constructs spanning a range of stabilities was made, and it was found that the most stable ones could function for inhibiting the antigen activity *in vivo* (15). In the present work, we first established a system for directly identifying scFvs that are well expressed, soluble, and stable enough to bind the antigen of interest in the absence of disulfide bonds inside a eukaryotic cell. In the second part we then extended this work to select for scFvs binding a different antigen. In our vectors scFvs were fused to the C terminus of the activation domain of Gal4. The model antigen, which consists of the leucine zipper domain of the yeast transcription factor Gcn4, was fused to the DNA-binding protein LexA (see under "Experimental Procedures"). Both hybrid proteins were expressed in the yeast strain YDE173 carrying the divergently oriented reporter genes *HIS3* and *lacZ* both under the control of six LexA-binding sites (Fig. 1). The *lacZ* gene allows quantification of reporter gene activity, whereas the *HIS3* gene can be exploited to identify antigen-antibody interactions by growth selection.

To develop and validate our system, several previously characterized scFv fragments displaying very similar antigen-binding properties, but different *in vitro* stability and *in vivo* performance (15), were fused to the activation domain (AD) of Gal4. Yeast cells co-expressing anti-Gcn4 scFv variants fused to the activation domain of Gal4 and with the leucine zipper antigen fused to LexA were grown in culture and tested by a β-galactosidase assay to quantify reporter gene activation (Fig. 1).

[4] C. Zahnd, B. Luginbühl, S. Spinelli, L. Jermutus, P. Amstutz, C. Cambillau, and A. Plückthun, manuscript in preparation.

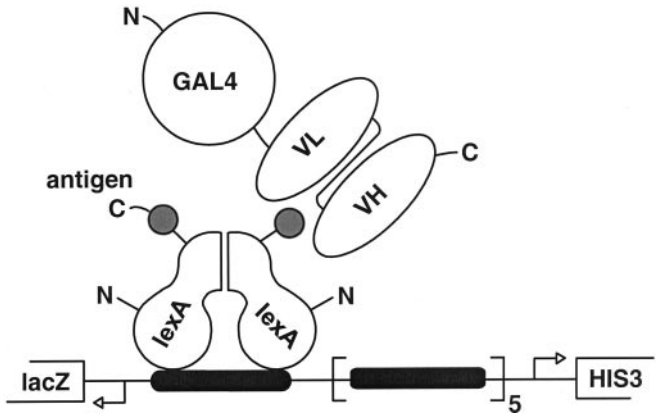

FIG. 1. **Schematic drawing of the modified yeast two-hybrid system for directly identifying stable intracellular scFvs that can bind an antigen inside a eukaryotic cell.** A single-chain Fv antibody fragment is linked to the transcriptional activation domain of Gal4 and the antibody-specific target antigen is fused to a DNA-binding domain (LexA). LexA forms a dimer as indicated (53). These constructs are co-transformed into the reporter yeast strain YDE173 carrying a stably integrated reporter construct. This composes the divergently oriented reporter genes *HIS3* and *lacZ* under the control of six LexA-binding sites (indicated by *black bars*). The scFv can mediate the specific interaction with the antigen fused to LexA. Thereby a complex is formed that can activate transcription of the reporter genes. Thus, a successful antigen-antibody interaction results in the expression of the *HIS3* gene and enables growth of yeast on selective plates. Additionally, reporter gene activation can be quantified by measuring β-galactosidase activity produced by the *lacZ* gene.

The original anti-GCN4 wild-type (anti-GCN4wt) scFv had been obtained by ribosome display from a murine library (26). Different variants of this anti-GCN4 single-chain antibody 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 (15). These anti-GCN4 scFv fragments were either point mutants of the original murine antibody, or the CDRs were grafted on a stable framework. Because the CDR donor had a λ light chain, but the recipient framework light chain was of κ type, two versions were constructed, one with the original κ V_L-V_H dimer interface residues ("κ-graft") and one with additional six dimer interface residues changed, which were not involved in antigen contact, to retain the original domain orientation ("λ-graft") (15).

Fig. 2*A* shows that the fusion protein bearing the stable scFv moiety called λ-graft exhibited the strongest effect in our screening and selection system, followed by the anti-GCN4wt, and the destabilized point mutant anti-GCN4(H-R66K), corresponding to position H-77 in the new nomenclature (20). In contrast, the even more stable but weakly binding κ-graft and the cysteine-free anti-GCN4(SS^-) variant of the wild-type framework caused no significant reporter gene expression. The control fusion protein bearing the unrelated scFv AL5, which is unable to interact with the Gcn4 leucine zipper, did not activate the *lacZ* reporter gene. The same results were obtained in a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plate assay (data not shown). In summary, the *in vivo* activity of the different Gal4AD-scFv fusion variants in our two-hybrid based screening system correlated well with the performance of these scFvs as intracellular inhibitors of Gcn4, as described previously (15).

Interaction between the Antigen and the Intrabody Mediates Growth of Yeast Cells on Selective Media—The reporter construct, which is integrated in the yeast genome, contains the *HIS3* gene under the control of six LexA-binding sites (Fig. 1). Binding of the scFv-Gal4 fusion to the antigen-LexA fusion is

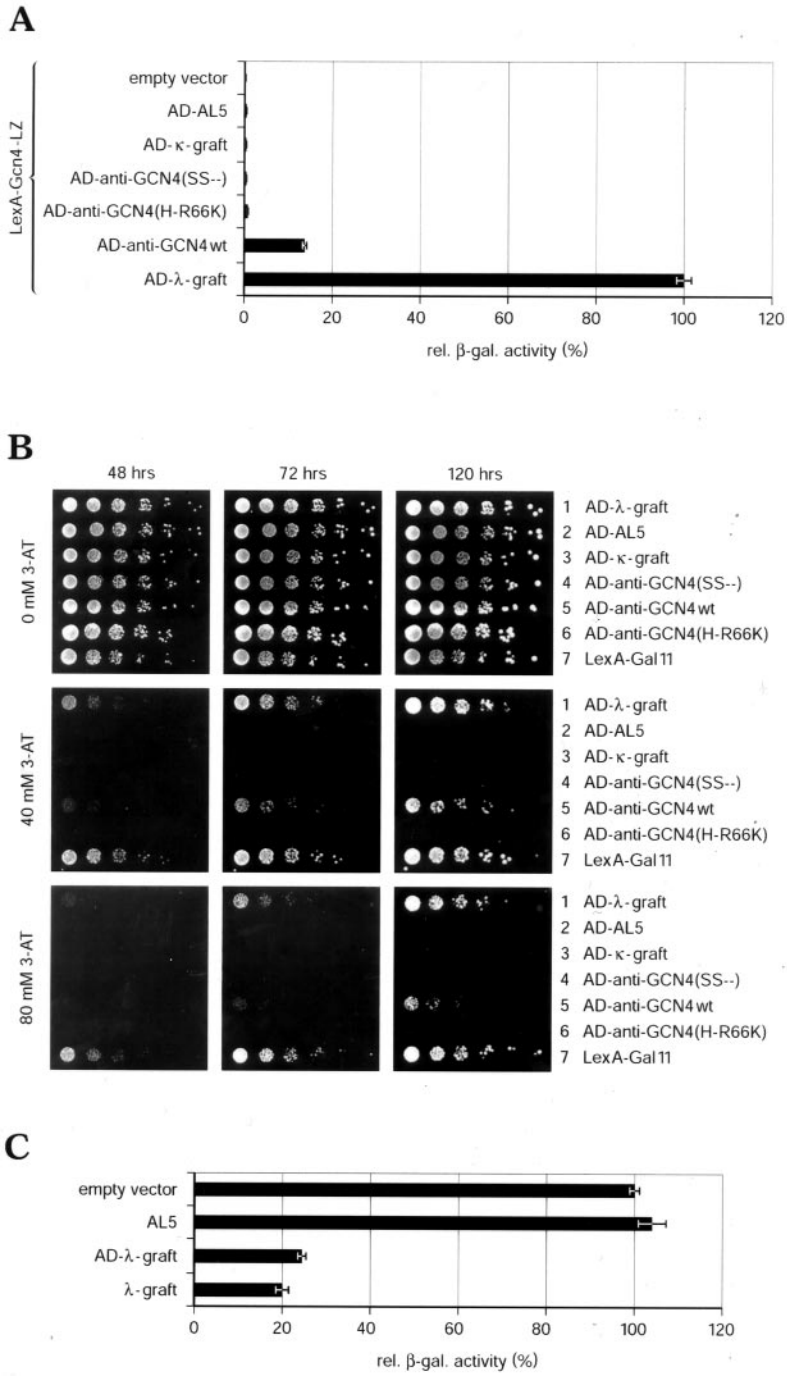

FIG. 2. **Intracellular interaction of scFv with target antigen results in reporter gene expression.** *A*, the yeast strain YDE173 was co-transformed with plasmids expressing the LexA-Gcn4 leucine zipper fusion and the various scFvs fused to the activation domain of Gal4 (Fig. 1) or an empty plasmid as indicated. A β-galactosidase (*β-gal*) assay was performed in solution to quantify reporter gene activation. Relative β-galactosidase activity obtained with the λ-graft fusion (*AD-λ-graft*) was arbitrarily set to 100%. The highly stable λ-graft variant was the most potent intrabody in the antibody-antigen interaction approach, followed by anti-GCN4wt. Expression of the less stable Gcn4 leucine zipper binding scFv variants (*AD-anti-GCN4H-R66K* and *AD-anti-GCN4SS–*) did not result in significant reporter gene activation. In contrast, the κ-graft (*AD-κ-graft*) has a highly stable and soluble framework but is characterized by a low binding affinity (micromolar range) and therefore did not lead to significant reporter gene activation. AL5 (*AD-AL5*) is an ampicillin-binding scFv and serves as negative control. *B*, growth on selective plates containing 3-AT correlates with the gene activation potential of the various scFvs. Transformants co-expressing the LexA-Gcn4 leucine zipper and an AD-scFv fusion variant (*lanes 1–6* as indicated) were spotted in a 5-fold serial dilution starting with 10,000 cells on appropriate selective plates lacking histidine and supplemented with 0, 40, or 80 mM 3-AT. Cells were kept at 30 °C, and growth was monitored over 48, 72, and 120 h as indicated. The strong activator LexA-Gal11 (18) (*lane 7*) and AD-AL5 (*lane 2*) were used as positive and negative control, respectively. No growth difference was observed on selective plates without any 3-AT (*0 mM 3-AT*). At concentrations of 40 and 80 mM 3-AT only the λ-graft (*lane 1*) and anti-GCN4wt (*lane 5*) were able to grow, with a clear growth advantage for the λ-graft. *C*, the inhibitory potential of constitutively expressed λ-graft or λ-graft fused to the Gal4 activation domain (AD-λ-graft) was compared in the yeast strain YAdM2xGCN4-150 (15). This strain carries a stably integrated reporter construct expressing the enzyme β-galactosidase under the control of two Gcn4-binding sites. The λ-graft scFv interferes with Gcn4 dimerization and reduces activation of the Gcn4-dependent *lacZ* reporter gene. AL5, which is an ampicillin-binding scFv, and His derivative (AD-AL5) serve as negative controls like the empty vector. λ-graft and AD-λ-graft showed no significant difference in the ability to inhibit the Gcn4-dependent gene activation.

expected to restore growth on media lacking histidine. Thus, cell growth should be usable as a selection assay to identify antigen-antibody interactions. Furthermore, a combined property of stability, solubility, and affinity of the antibody for the antigen are expected to correlate with the level of gene activation that the interaction between these proteins can elicit, thereby allowing a selection for the "quality" of an intrabody. Various levels of *HIS3* gene activation can be readily monitored by selective growth in the presence of different concentrations of 3-aminotriazole (3-AT), a competitive inhibitor for the *HIS3* gene product.

The yeast growth selection experiments were performed by spotting yeast cells in a series of 5-fold dilutions, starting with ~10,000 cells on selective plates lacking histidine and containing different concentrations of 3-AT. Growth of yeast cells co-expressing the Gcn4 leucine zipper-LexA hybrid together with various Gal4AD-scFv fusion proteins was monitored over 5 days (Fig. 2*B*). Cells expressing the fusion protein LexA-Gal11 (18), a strong activator, were used as a positive control for strong *HIS3* activation and efficient growth. As a negative control, the unrelated scFv AL5 fused to the Gal4 activation domain was used. Under non-selective conditions, no difference in the growth rate could be observed between the positive control and any of the other transformed yeast cells, indicating that none of the constructs was toxic to the cell.

Under selective conditions, however, a 40 mM 3-AT concentration was sufficient to readily discriminate between cells expressing different fusion proteins (Fig. 2*B*). In agreement with the results obtained by measuring expression of the *lacZ* reporter gene (Fig. 2*A*), *HIS3* gene activation of the fusions with the scFv moieties of the κ-graft, the anti-GCN4(SS^-), and the less stable anti-GCN4(H-R66K) were insufficient to allow growth in the presence of 40 mM 3-AT. Even at concentrations of 20 mM 3-AT, growth of these cells was suppressed (data not shown). In contrast, cells expressing the λ-graft fusion or the anti-GCN4wt fusion were able to grow in the presence of up to 80 mM 3-AT. The most stable scFv framework, the λ-graft, clearly conferred a growth advantage to the cells under selective conditions, in particular after 72–120 h of incubation on plates containing 80 mM 3-AT (Fig. 2*B*). These results show that the *in vivo* approach presented here is a powerful tool to discriminate between antigen-specific single-chain antibodies that are functional in the reducing eukaryotic intracellular environment and those that have lost activity.

Fusion of the Gal4 Activation Domain to the λ-Graft scFv Does Not Interfere with the Original Biological Effect of the Antibody—For the intrabody selection system presented here, all scFvs were expressed as hybrid proteins fused to a transcriptional activation domain (Fig. 1). To test whether the addition of this N-terminal domain might influence the properties of the intrabody, we compared the original λ-graft scFv with the same single-chain antibody fused to the Gal4 activation domain for their ability to inhibit gene activation by the Gcn4 transcription factor as described previously (15). In this case, the reporter gene is controlled by Gcn4. Fig. 2*C* shows that the Gal4AD-λ-graft fusion variant reduced GCN4-dependent reporter gene expression to the same extent as the λ-graft alone, whereas both a nonspecific scFv (AL5) and the empty vector did not influence the GCN4-dependent gene expression. Thus, fusion of a heterologous domain such as the Gal4 activation domain to the scFv did not impair the biological effect of this single-chain antibody.

The in Vivo Performance of an Intrabody Can Be Optimized by Random Mutagenesis and in Vivo Selection—Our results show that only some of the anti-GCN4 scFv variants can bind the antigen in the reducing intracellular environment, where

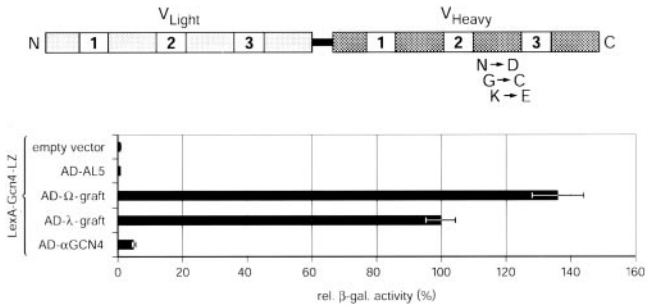

FIG. 3. **Framework mutagenesis and selection for improved *in vivo* performance.** The λ-graft framework was randomly mutagenized and screened for mutants with an improved intracellular performance in the antibody-antigen interaction approach with the Gcn4 leucine zipper fused to LexA. Selection was performed on plates lacking histidine and supplemented with 75 mM 3-AT. The focus was set on clones displaying faster growth and higher β-galactosidase (*β-gal*) activity than the λ-graft control. In this screening, three independent point mutations in the heavy chain (N84D, G85C, and K86E) were identified in three adjacent positions, which resulted in increased reporter gene activation. These mutations were combined into a single molecule named Ω-graft, which caused about 40% higher reporter gene activation than the parental λ-graft. For a structural interpretation of these mutations, see Fig. 4.

the stability-conferring disulfide bonds do not form. The ability of these scFv variants to function inside the cell correlates with the degree of stability and solubility of their frameworks and with a sufficient affinity for the antigen (15). We tested whether the *in vivo* approach presented here can be exploited to select for better performing intrabodies upon *in vitro* mutagenesis of an existing scFv. The already stable λ-graft variant was mutagenized by error-prone PCR in order to introduce amino acid changes spread all over the framework. This randomly mutagenized framework library was fused to the Gal4 activation domain. The scFv library was co-transformed together with the plasmid expressing the target antigen (Gcn4 leucine zipper) fused to LexA into the reporter yeast strain YDE173. Transformants were grown on selective plates containing 75 mM 3-AT to identify candidate clones showing an improved growth behavior compared with the λ-graft. Colonies that grew faster than the λ-graft-expressing cells were further tested for higher reporter gene expression by measuring β-galactosidase activity. Sequence analysis of 8 selected clones indicated that they harbored one or more point mutations, mostly in the framework but a few also in the CDR sequences. Some of the mutations showed up several times in different combinations. Three framework mutations, which were found in 4 independent clones and are adjacent to each other on the outer loop of V_H, appeared to give the highest activity in the interaction assay (data not shown) and were combined into one framework. This framework with three point mutations was given the nickname Ω-graft. It displayed an almost 40% better *in vivo* activity than the original λ-graft in the interaction assay as monitored by measuring the *lacZ* expression (Fig. 3).

While the present study was being carried out, the x-ray structure of the wt GCN4 scFv (26) in complex with the antigenic peptide[4] as well as that of the framework donor, a hyperstable hybrid scFv of 4D5 and AB48 (29), was determined.[4] This allowed us to build a structural model of the λ-graft and the Ω-graft with bound antigen (see under "Experimental Procedures"). The three mutations, which were responsible for the beneficial effect *in vivo*, were all found to be in the outer loop of V_H (Fig. 4) having their side chains exposed. They are too far away from the antigen to make direct contact. This indicates that the improved behavior is not an affinity effect but could in principle be evoked by a higher solubility, expression rate, *in vivo* stability, or a combination thereof. The findings portend

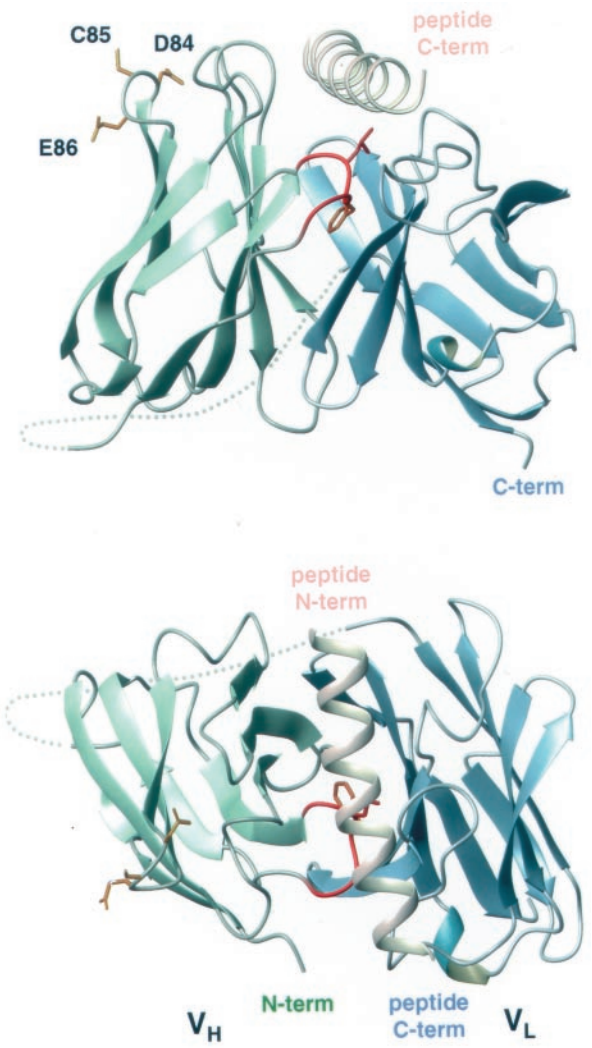

FIG. 4. **Structural model of the Ω-graft framework variant ALF.** The model was built based on the crystal structure of the wild-type GCN4-binding scFv in complex with its antigen and the crystal structure of the framework donor[4] (29). The Fv portion is shown in a ribbon representation with its helical antigen on the *top.* The heavy chain is drawn in *light green,* and the light chain is drawn in *light blue.* The linker connecting V_H and V_L is indicated by the *dotted line.* The framework residues changed compared with the λ-graft are colored *orange.* They all lie in the outer loop of V_H and appear to influence the folding efficiency of scFv rather than the intrinsic protein stability or binding affinity. The complementarity-determining region 3 of V_H (CDR-H3) and the side chains of the three residues, which were randomized, are colored *red*. The figure and the model were generated using the program MolMol (54).

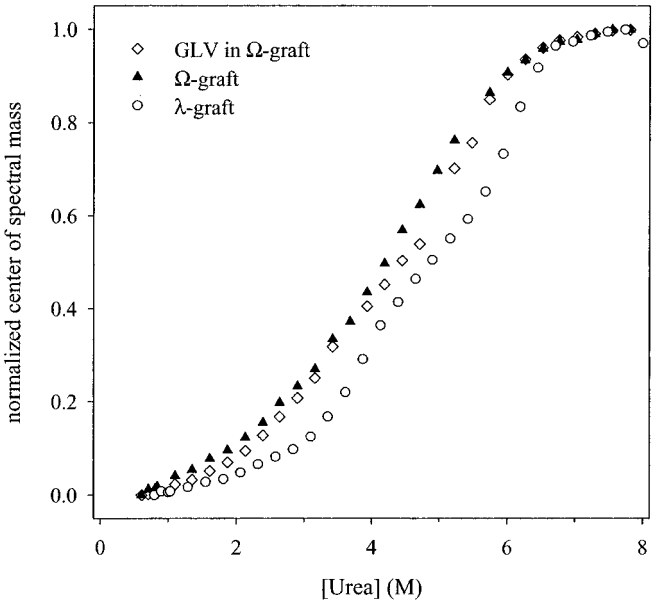

FIG. 5. **Urea-induced equilibrium refolding of different Gcn4-binding single-chain antibody fragments under reducing conditions.** The center of spectral mass was normalized for the pre-transition and the post-transition base line. Shown are the λ-graft (○), Ω-graft, which showed an improved *in vivo* performance (◇), and a variant of Ω-graft bearing the sequence GLV in the CDR-H3 (▲). In the λ-graft two transitions can be distinguished, whereas both variants of the Ω-graft framework show a broad transition. As none of the curves shows a two-state transition, it is impossible to calculate thermodynamic stabilities. Nevertheless, the concentration of urea at which the protein starts to refold can be taken as a measure of its relative stability. The most stable protein *in vitro* is the λ-graft. The two variants of Ω-graft, differing in CDR-H3, both exhibit the same stability within the error of the measurement. However, they are both less stable than the λ-graft *in vitro*.

that the selection method can be used to identify variants with improved properties within eukaryotic cells. In order to better understand the molecular parameters selected, we expressed the Ω-graft in *E. coli* and characterized it *in vitro*.

The Improved in Vivo Performance of the Selected scFv Is Due to Other Properties Than Higher Thermodynamic Stability or Affinity—We tested whether the faster growth of the yeast cells under selective conditions and the higher activity of the β-galactosidase gene was because of higher intrinsic stability of the single-chain Fv fragments. Therefore, we cloned the λ-graft, the Ω-graft, and a CDR-H3 mutated version of Ω-graft, where three residues had been changed to the sequence GLV (see below), without the activation domain into a periplasmic *E. coli* expression vector allowing the formation of the disulfide bonds of the protein. The intrinsic stability of the overexpressed and purified single-chain antibody fragments was first measured by denaturant-induced equilibrium unfolding and refolding without prior reduction. The equilibrium denaturation curves induced by guanidine hydrochloride denaturation of the Ω-graft and the GLV variant of Ω-graft in the oxidized state looked exactly the same, indicating that the sequence change in the CDR-H3 region did not influence the stability of the whole protein, whereas the curve of λ-graft showed a biphasic curve, shifted to higher concentrations of guanidine hydrochloride (data not shown). Because none of the single-chain Fv fragments showed a two-state transition of the unfolding process, which makes it impossible to calculate values for the free energy (15), the relative stabilities of the investigated clones were compared. Neither the Ω-graft nor its CDR-H3 variant GLV showed an improved stability compared with the λ-graft. The free cysteine introduced at position H-85 in the Ω-graft may contribute to the broad unfolding transition of the protein under non-reducing conditions by forming mixed disulfides in the transition region.

In order to use an experimental set up that better simulates the conditions of the selection in an intracellular environment, we performed the stability measurements under reducing conditions with urea as denaturant (Fig. 5). Because it might be difficult to reduce the intradomain disulfide bond in the native state of single-chain Fv fragments (30), we decided to unfold the proteins first, making the disulfides accessible to reduction at high pH, and then measure the unfolding equilibrium of the reduced protein starting the reaction from unfolded protein. The curves of the urea-induced unfolding equilibrium of the reduced protein closely reflected the observations in the oxidized state. The curves of the Ω-graft and the GLV variant of the Ω-graft looked the same within the error of the measure-

ment. As in the oxidized state, the transition state of both clones was shifted to lower denaturant concentration than the one of the λ-graft, indicating them to be less stable proteins (Fig. 5). These results indicate that the selection has favored other properties than stability, such as, for example, expression rate, solubility, affinity, and resistance to proteases. However, a stability threshold must be surpassed for showing biological activity, as shown in Fig. 2.

Direct Intracellular Screening to Identify Novel CDRs Interacting with the Antigen—In a wealth of structural studies on antigen-antibody interactions it was found that residues in the complementarity-determining region 3 (CDR-H3) of the heavy chain generally contribute very important contacts to the antigen (31–33). This was also seen in the x-ray structure of the protein in complex with the peptide[4] (Fig. 4). We tested whether the *in vivo* approach presented here might be exploited to directly isolate antigen-binding scFvs by screening a library of randomized CDR-H3 sequences. The CDR-H3 of the anti-GCN4 intrabodies is very short and consists of only 5 amino acids (GLFDY) (15). To construct a library, we used the Ω-graft framework as scaffold, in which the first three amino acids (GLF) of the V_H CDR3 were randomized, whereas the next two residues (Asp-137 and Tyr-138) were kept constant, because their structural importance was demonstrated in many cases (32). The library was subjected to the selection system described here to test if the original as well as perhaps similar or novel CDR-H3 sequences could be identified that bind the Gcn4 antigen *in vivo*.

The reporter yeast strain YDE173 was transformed with vectors expressing the antigen (Gcn4 leucine zipper) fused to LexA together with the random CDR-H3 scFv library fused to the Gal4 activation domain. A total number of 250,000 clones was screened on selective plates containing 75 mM 3-AT. After 3 days, ~1500 colonies appeared on these plates. Fifty clones were picked, and the library plasmids were isolated and retransformed into the same reporter yeast strain to confirm antigen-dependent gene activation. This test showed that 84% of the originally isolated clones activated the *lacZ* gene in the presence of the antigen fusion protein, whereas 16% did not. The heavy chain CDR3 region of these positive clones was sequenced. In addition to the original residues in CDR-H3 (GLF), 13 novel CDR-H3 sequences were identified (Fig. 6). Several CDR-H3 variants were found more than once and showed different codon usage (*e.g.* GLV was found 7 times and glycine was encoded by GGG or GGT, leucine by TTG or CTG, and valine by GTG or GTT), underlining that the selection was really for the amino acid sequence. The CDR-H3 variant containing the amino acid composition GFA, which was randomly picked from the unselected library, was neither able to bind the antigen (see below) nor could it activate the reporter gene. In summary, these results demonstrate the feasibility of an intracellular screen, to select for the binding activity of antibodies. The close relationship to the original sequence, which can be rationalized by the structure, the use of different codons for the same protein sequence, and the affinities (see below) suggest that a functional selection for antigen binding has taken place.

The Selected CDR-H3 Sequences Maintain High Affinity for the Antigen—In order to estimate the relative affinities of different clones selected from the CDR-H3 randomized library, a competition RIA was performed. Four variants of the Ω-graft carrying the sequences GLF (original Ω-graft), ALF, GLV, and GLW within their heavy chain CDR3 region (see Table I) were compared with the previously characterized λ-graft, also carrying the original sequence GLF. As a negative control, a clone carrying the sequence GFA was used, which was not able to activate transcription *in vivo* (see above). All constructs were transcribed *in vitro*, and subsequently, equal amounts of mRNA were translated *in vitro* in an S30 *E. coli* translation system containing [^{35}S]methionine as the only methionine source (see "Experimental Procedures"). It was verified with a radioimmunoblot that the amount of protein produced *in vitro* was the same for all the constructs (data not shown).

	CDR3 sequences						rel. β-gal. activity (%)	
Kabat	*94*	*95*	*96*	*97*	*101*	*102*		
AHo	*108*	*109*	*110*	*136*	*137*	*138*		
λ-graft	T	**G**	**L**	**F**	**D**	**Y**	**100**	**± 5.3**
Ω-graft		G	L	F			136	± 19.5
Sequence variation in CDR-H3		**G**	**L**	**V**			**58**	**± 4.4**
		G	**L**	**W**			**136**	**± 11.7**
		G	L	L			126	± 18.1
		G	**L**	**F**			**142**	**± 18.5**
		G	L	C			93	± 17.8
		G	L	H			102	± 5.7
		G	L	M			131	± 13.7
		A	L	W			114	± 14.1
		A	**L**	**F**			**92**	**± 2.4**
		G	L	Q			50	± 3.0
		G	L	Y			115	± 5.1
		G	V	M			133	± 13.6
		G	V	L			64	± 2.1
		G	V	C			91	± 2.0
		G	V	F			91	± 4.8
		G	I	W			106	± 8.1
		G	**F**	**A**			**1.8**	**± 0.2**

FIG. 6. **CDR3 V_H chain screening for testing the range of sequences able to bind the leucine zipper of Gcn4.** Reporter gene activation of the different CDR-H3 variants was determined by measuring β-galactosidase activities in the yeast strain YDE173. Selected CDR-H3 sequences (V_H 109–136) are listed with the corresponding β-galactosidase activity. The aspartate at position V_H137 and tyrosine at position V_H138 were not randomized (see also Fig. 8). Relative β-galactosidase activity obtained with λ-graft was set to 100% (*top line*). All other given CDR-H3 sequences were in the Ω-graft framework. GFA is a clone that has lost the ability to bind the antigen and thus failed to activate the β-galactosidase reporter. The activation potential of almost all CDR3 V_H variants is comparable with the one observed with the λ-graft. Clones indicated in **bold** were analyzed in a RIA for their binding properties (Fig. 7).

The ability of low concentrations of soluble antigen to inhibit binding of the single-chain Fv fragments to surface-immobilized peptides was taken as a measure for the relative affinity of the clone. In the presence of 10^{-7} M soluble Gcn4, the binding of all clones investigated was inhibited to more than 90%. The clone carrying the sequence GFA, which did not interact with the antigen in the cellular assay, did not bind the immobilized antigen above background (data not shown). For a comparison of the relative affinities, competitive inhibition at $1.5 \cdot 10^{-8}$ M of free antigen was monitored (Fig. 7*A*). The λ-graft carrying the sequence GLF in the CDR-H3 was most efficiently inhibited, indicating the highest affinity of the investigated clones. The Ω-graft itself was slightly less inhibited with 38%. Inhibition could still be observed for the Ω-graft variants carrying the sequences GLW and GLV, which showed 74 and 86% of the uninhibited signal, respectively. Clone ALF could not be inhibited by this concentration of free antigen at all.

Next, we determined the dissociation constants of three single-chain Fv fragments using competitive inhibition BIAcore as described (26). Purified λ-graft protein and two of the Ω-graft variants, GLW and ALF, were measured. The dissociation constant K_D of λ-graft was $3.5 \cdot 10^{-10}$ M, which is in good agreement with previous measurements (15). Confirming the RIA experiments, the affinities of the two Ω-graft variants were slightly lower. The GLW variant had a 2-fold higher K_D of $6 \cdot 10^{-10}$ M and the ALF variant a 3-fold higher K_D of $1.1 \cdot 10^{-9}$ M (Fig. 7*B*).

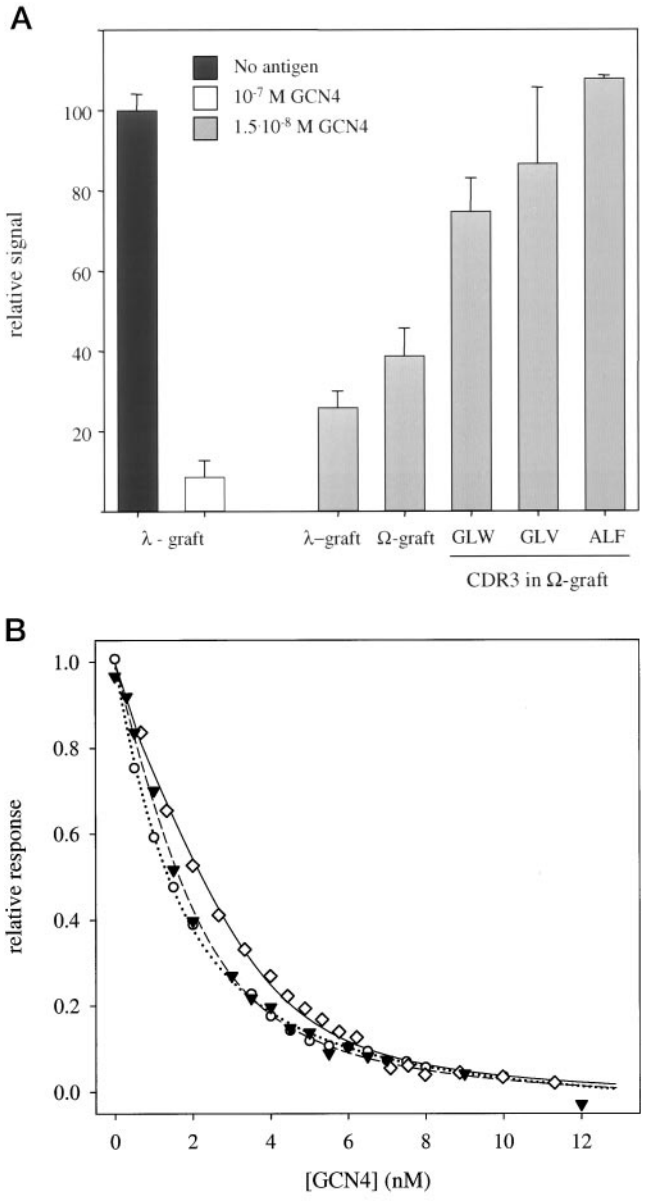

FIG. 7. **Affinity determination of different Gcn4-binding clones.** The affinities of different Gcn4-binding variants of the λ-graft and Ω-graft were measured using a radioimmunoassay and inhibition BIAcore. For the radioimmunoassay (*A*), the λ-graft and several CDR-H3 mutants of Ω-graft were translated *in vitro* in the presence of [^{35}S]methionine. The radioactively labeled protein was bound to surface-immobilized antigen, both in the presence and in the absence of soluble antigen as competitor. The binding signal to the surface-immobilized antigen in the absence of competitor antigen was normalized to 100% signal (*dark gray bar*). The extent of inhibition of the signal in the presence of small amounts of antigen correlates with the affinity of the scFv to the antigen. At 10^{-7} M antigen, the signal was inhibited to more than 90% for all constructs (only shown for λ-graft, *shaded bar*). The signal in the presence of $1.5{\cdot}10^{-8}$ M antigen was used to estimate relative affinities (*light gray bars*). The highest affinity is shown by the λ-graft. All CDR-H3 variants of Ω-graft seem to have lowered the affinity of the scFv. *B,* binding affinities were measured using inhibition BIAcore. λ-graft (◇) and two variants of the Ω-graft framework, GLW (▼) and ALF (●), were measured. The dissociation constant K_D of λ-graft was determined to $3.5{\cdot}10^{-10}$ M, the one of GLW to $6{\cdot}0.10^{-10}$ M, and the one of ALF to $1.1{\cdot}10^{-9}$ M.

The selection showed a strong conservation of the structural features in all three positions. A closer look at the CDR3 sequence of the heavy chain (positions H-109, H-110, and H-136 (20)) in the structural model, based on the crystal structure of the CDR-donor[4] and the framework donor,[4] shows that glycine H-109 is part of a two-residue β-hairpin (Fig. 4). Glycines are highly conserved in this structural motif for steric reasons (34, 35), explaining the lower affinity of the alanine mutant ALF. At position H-110, leucine or at least valine or isoleucine consistently showed up. The side-chain is tightly packed with hydrophobic residues being contributed both by the scFv and the antigen. The direct contact to the antigen may be the prime reason for the strong conservation of the residue. Residue H-136, which showed the weakest conservation after selection, does not make any contact to the antigen. The side-chain points into the framework of V_H and is enclosed by two tryptophan residues. The relatively loose packing of this hydrophobic pocket may explain the tolerance for substitutions at this position.

Even if the selection did not improve the absolute affinity of the single-chain fragment, the selected spectrum of amino acids and the similar K_D values showed selection for residues allowing this type of interaction. These results indicate that the system tolerates a spectrum of affinities, as long as they lie above a certain threshold value.

Direct Intracellular Selection of Intrabodies against the Oncoprotein c-Jun—The leucine zipper is a common dimerization motif and occurs in several DNA-binding proteins. In addition to the leucine zipper of the yeast transcription factor Gcn4, the products of the mammalian proto-oncogenes *jun, fos,* and *myc* also carry this module (36, 37). Fig. 8*A* shows an alignment of the leucine zipper domains of Jun and Gcn4. We wanted to examine whether intrabodies can be selected that specifically bind the Jun leucine zipper *in vivo*. A screen of the library described above did not yield any binder specific for the Jun leucine zipper. We therefore constructed a new library based again on the Ω-graft intrabody framework, which carried 8 fully randomized positions within a 10-amino acid long heavy-chain CDR3 sequence. The last two residues (DY) were again kept constant. This library was screened *in vivo* against the leucine zipper of the Jun protein fused to LexA. A total number of 20 million clones were screened on selective plates containing 10 mM 3-AT. Colonies that appeared between day 3 and 5 were streaked on selective plates for another round of growth selection. Plasmid DNA of 44 candidate clones was isolated and retransformed into the same reporter yeast strain expressing either the leucine zipper of Jun or the leucine zipper of Gcn4 fused to LexA. Forty one clones (93%) were able to activate the *lacZ* gene in the presence of the Jun leucine zipper fusion protein but not with the one of Gcn4. The remaining three clones no longer showed any activity irrespective of the type of antigen (Fig. 8*B*). The selected CDR sequences showed a tendency for two different consensus sequences. They preferentially contained a proline at the first position of the randomized 8-mer in combination with a lysine and phenylalanine at positions 3 and 4. Alternatively, a proline showed up in position 6 in combination with an arginine in position 8 (Fig. 8*B*).

Even though the Jun antigen and the Gcn4 leucine zipper are structurally related, it is likely that the scFv binds the antigen in a different way. From the structure of the original complex (Fig. 4), it is clear that the original 5-amino acid long CDR-H3 loop is underneath the helical peptide. Of the two conformations found most prominently in the protein structure data base for CDR-H3 loops of 10 residues (exemplified by the structures PDB 1CLO and 1C1E, respectively), one clearly clashes with the antigen, whereas the other would at least require a significant shift in the antigen.

These results indicate that CDR libraries constructed on intracellular stable scFv frameworks can be directly screened *in vivo* to identify intrabodies for binding activity.

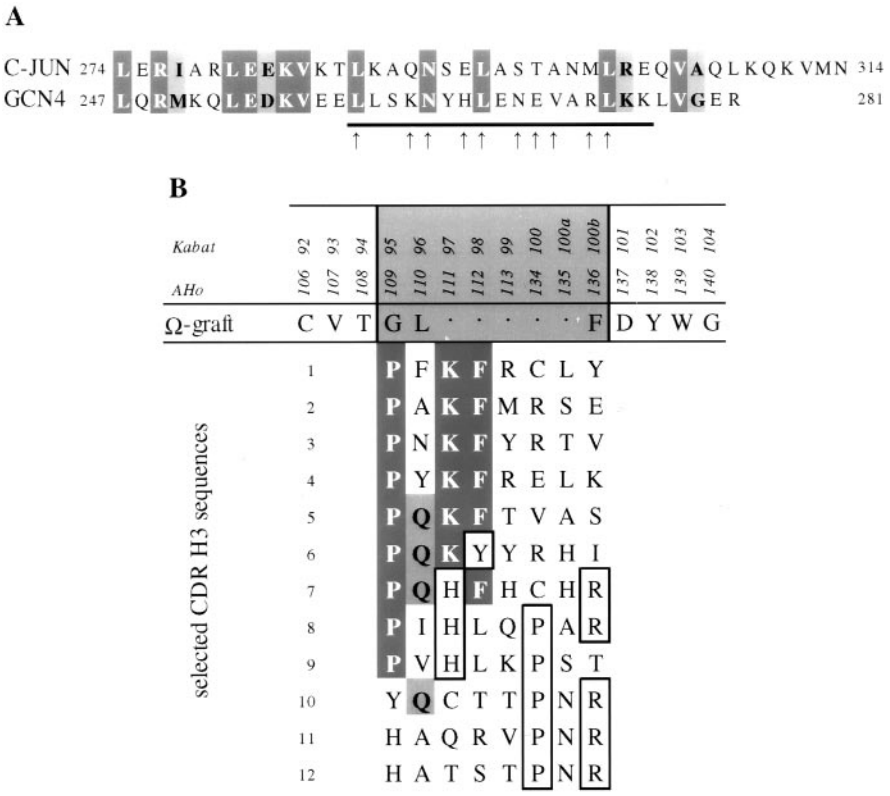

FIG. 8. **Isolation of Jun-specific intrabodies by screening a CDR3 V_H 8-mer library constructed on a defined framework.** *A*, protein sequence alignment of the leucine zipper domains of the human Jun protein and the yeast transcription factor Gcn4. Identical residues are shown as *white letters* on *dark gray*, and similar amino acids are on a *light gray background*. Note that both domains share very little amino acid homology except the structurally important leucine residues seven amino acids apart. The part of the peptide, binding to the single-chain antibody Fv fragment, is *underlined*. *Arrows* indicate contacting residues. *B*, CDR-H3 amino acid sequences (V_H 109–138) of the scFv antibody fragments isolated after a selection for the leucine zipper of the human Jun protein. Only residues V_H 109–136 were randomized. Apparent consensus residues are *highlighted*. The optimal amino acid sequence in the first half of the randomized 8-mer appears to be P(*X*/Q)KF (indicated with *black* and *dark gray background*), and for the last four residues the common motif is *X*P*X*R (framed). Note, however, that these two motifs never appear together.

DISCUSSION

The potential of single-chain antibody fragments (scFvs) for intracellular applications is currently being recognized, in particular in the field of functional genomics (38). These so-called intrabodies have generally been derived either from specific monoclonal antibodies or from single-chain antibody fragments that were first selected by *in vitro* techniques, such as phage or ribosome display, and subsequently tested for their biological activity within eukaryotic cells (15, 39, 40). Both methods do not automatically ensure that the isolated antibodies do also bind their cognate antigen in the cytoplasm of a cell, which needs to be established in a separate test. Indeed, the cytoplasm is a non-physiological environment for the antibodies, and due to the reducing conditions that prevent the formation of disulfide bonds, most scFvs have been found to be inactive inside eukaryotic cells (41, 42). A technology to directly select antibodies under reducing conditions is thus required. Additionally, frameworks are needed that are sufficiently robust to allow folding under reducing conditions.

In this study, we present a system to directly select active intrabodies in yeast. This system is based on the yeast two-hybrid assay (17) and couples the expression of reporter genes to an antibody-antigen interaction. The expression level of the reporter genes can conveniently be monitored, allowing an efficient screen by yeast cell growth selection. To test our system, we used a set of scFv fragments displaying the same specificity for one epitope, namely the leucine zipper of the yeast transcription factor Gcn4, but showing different *in vitro* stabilities as well as *in vivo* performances (15). The scFv variant with the highest activity in our system was the so-called λ-graft (as it used V_L-V_H dimer interface residues in a κ-framework taken from its λ CDR donor). It was also the least aggregation-prone and most stable single-chain antibody *in vitro* (15). In addition to folding efficiency and stability, the affinity for the antigen must surpass a threshold value for specific detection of intracellular function in our system. This requirement was demonstrated with the κ-graft variant, which retained the κ dimer-interface residues in the κ-framework and contains the most stable framework, but was nevertheless unable to activate reporter gene expression due to its low affinity for the Gcn4 antigen (15). We found that the results obtained with our intracellular interaction assay (Fig. 2) correlated well with those from our previous biochemical and functional analyses, in which various anti-GCN4 scFv variants exhibited different abilities to inhibit the Gcn4 activity *in vivo* according to their stability and their affinity for the antigen (15). Our data underline that the *in vivo* performance of intrabodies displaying the same antigen specificity can vary dramatically according to the degree of stability of the antibody framework. To efficiently exploit the high degree of antigen specificity and affinity of antibodies for intracellular applications, it will be necessary to use antibody frameworks that have been optimized for intracellular stability and solubility. In principle, there are two approaches to obtain a more stable framework, namely by protein engineering (15, 43) or by directed evolution (42, 44, 45).

In the present work, we isolated an scFv framework from a library generated with error-prone PCR that behaved better *in vivo*. The results of our *in vitro* analysis point out that this beneficial effect was not due to improved thermodynamic stability or affinity and that other properties of the single-chain Fv fragment, such as its solubility and expression rate or its resistance to proteases, have probably been optimized. Because the selection system allows a straightforward selection for intrabodies directly under the relevant *in vivo* conditions, properties important for subsequent intracellular application may be optimized this way.

Furthermore, we show that our system is suited to select antigen-binding intrabodies from a library of randomized CDR-H3 sequences embedded in a stable framework (Fig. 6 and Fig. 8). In a previous report (46), we have presented a method for the direct *in vivo* testing and selection of intrabody frameworks independently of their antigen-binding specificity. These pre-selected frameworks can evidently

serve as backbones to construct intrabody libraries by randomization of hypervariable loops, which can be screened by the system presented here to identify specific binders. The possibility to use growth selection should also enable identification of binders with improved overall performance (expression, stability, or affinity) upon randomization of additional sequences. With the screen of one library described in this work, we identified a range of CDR-H3 sequences capable of recognizing the Gcn4 leucine zipper (Fig. 6) with an affinity that is in the same order of magnitude as that of the original CDR-H3. Moreover, by screening a larger library, in which the original three amino acids CDR-H3 were replaced by a longer loop carrying eight randomized positions, we were able to identify several intrabodies that specifically bound the leucine zipper of the human oncoprotein Jun (Fig. 8). The selected intrabodies suggested two possible consensus sequences in the randomized CDR-H3, but the characterization of these proteins will require a more detailed investigation.

In previous reports, the two-hybrid approach for detecting antigen-antibody interactions has mainly been used to identify sufficiently stable binders from a pool of scFvs, which had been first selected by antigen-specific phage display from large libraries (39, 47, 48). This double-step approach was necessary because only a fraction of a typical scFv library will generally have sufficient stability for intracellular application, and yeast cannot be readily used to screen very large libraries. The use of an intracellular *E. coli* selection system for antibody-antigen interactions, the protein complementation system, has recently been described with a model system (14). In one example of direct screening in yeast with a small library of $3.6 \cdot 10^6$ members, only a single sequence against the target was indeed found (40). In our approach, we have used a library based on a stable framework from the outset. Even though only one CDR was randomized in the present example, several binders against a new target could be selected, and a variation around consensus sequences was observed. One additional characteristic feature of our approach is that, contrary to *in vitro* panning or animal immunization, no antigen purification was required to identify antibodies or select pool of binders. Indeed, with the system described here, genes or gene fragments can be directly expressed in yeast to identify antibodies that specifically target their protein products.

A major application for intrabodies lies in functional genomics. As more and more genomes become sequenced, the number of novel proteins with unknown function steadily increases. Intrabodies provide an attractive tool to characterize the function of these gene products and to validate them as potential drug targets. Intrabodies can be expressed in a spatially and temporally controlled manner and be directed against unique protein domains. This great selectivity allows modulation of the function of one domain by the specific intrabody at any time without interfering with other activities of the target protein (49). Such *in vivo* "protein domain knockout" can mimic the action of putative specific drugs more closely than by depleting cells of the whole protein. Furthermore, an intrabody that binds a motif common to a protein family might be exploited to modulate the function of the entire protein family. These advantages of the intrabody technology can be used to complement other functional genomic techniques such as gene knockout (50), antisense (51), and RNAi (52). Therefore, protein or protein domain knockout through the intracellular expression of neutralizing scFv molecule represents an attractive approach to study the function of proteins.

In summary, to employ intrabodies as a readily available commodity in functional genomics, selection and identification of binders for a specific target of interest *in vivo* have to be reliably performed. With the model system described here we provide evidence that a direct intracellular screening can be performed to identify novel CDRs interacting with the antigen of interest.

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