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COMMUNICATION

Fast Selection of Antibodies without Antigen Purification: Adaptation of the Protein Fragment Complementation Assay to Select Antigen-Antibody Pairs

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We have adapted the protein fragment complementation assay (PCA) to the screening and selection of antibodies in the single-chain Fv (scFv) format. In this assay, two interacting proteins (target and antibody) are genetically fused to the two halves of the dissected enzyme dihydrofolate reductase. Binding of the two partners reassembles this enzyme and reconstitutes its activity, thus allowing growth on minimal medium. We have optimized this system with regard to linker length and orientation, and can reach an efficiency for antigen/antibody interactions similar to that with fused leucine zippers. Using several model antibodies specific for peptides and proteins, we show that cognate interactions give rise to about seven orders of magnitude more colonies than non-specific interactions. When transforming mixtures of plasmids encoding different antigens and/or antibodies, all colonies tested contained plasmids encoding cognate pairs. We believe that this system will be very powerful as a routine system for generating antibodies, especially in functional genomics, since it does not require purification and immobilization of the antigen. The identification of an antibody specific for a cDNA or EST-encoded protein will require only cloning, transformation and plating of bacteria.

Keywords: intrabodies; protein fragment complementation assay; trimethoprim, *in vivo* selection; functional genomics

The fast rate of gene discovery by several large-scale genome projects has generated an incredibly large amount of sequence data, which are, however, not matched by equivalent functional data. The availability of monoclonal antibodies is often a limiting prerequisite for carrying out functional studies on the newly discovered genes. Several techniques are available that allow screening of large antibody libraries for the identification of specific binders. All of these methods have in common that the genetic information for the protein of interest is physically linked to its phenotype, and the genes of selected antibodies can be reamplified and are readily available for further analysis. This spectrum of screening and selection techniques includes, among others, phage display (Smith, 1985; Winter *et al.*, 1994), ribosome display (Hanes *et al.*, 1998), surface display on bacteria and yeast (Boder & Wittrup, 1997; Georgiou *et al.*, 1997), and the yeast two-hybrid system (Fields & Song, 1989; Uetz *et al.*, 2000).

In order to develop a system that would be very fast and not prone to false positive results, a selection technique was developed that is based on the functional complementation of the bacterial enzyme dihydrofolate reductase (DHFR) with its murine counterpart (mDHFR), which has been called protein fragment complementation assay (PCA) (Pelletier *et al.*, 1998, 1999). In PCA, the gene of mDHFR is dissected into two parts (fragment I and fragment II, see Figure 1), each of them fused

E.M. and H.K. contributed equally to this stydy.

Abbreviations used: HAG, peptide of hemagglutinin; PCA, protein fragment complementation assay; scFv, single-chain F_v; TMP, trimethoprim; DHFR, dihydrofolate reductase; GCN4, transcription factor for the general control of amino acid biosynthesis.

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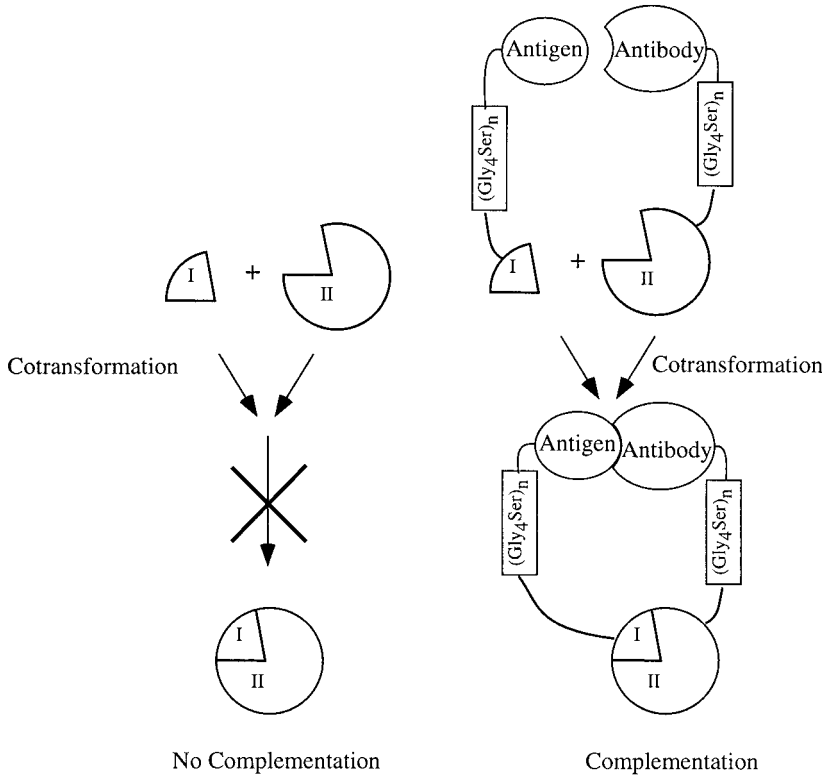

Figure 1. Principle of the protein fragment complementation assay (PCA). The enzyme DHFR from mouse (mDHFR) is genetically dissected into two fragments, named I and II. Active mDHFR allows *E. coli* to grow on minimal medium in the presence of the antibiotic trimethoprim (TMP), which inhibits the bacterial DHFR. The fragments are encoded on separate plasmids. Cotransformation of both plasmids and subsequent expression of the fragments by themselves in the bacterial cytoplasm does not lead to functional complementation of DHFR. However, fusion of a linker and an antigen to fragment I of mDHFR and a linker attached to the respective antibody to fragment II leads to functional complementation of DHFR and therefore to the growth of *E. coli* on minimal medium in the presence of TMP.

to a protein or peptide that can either homo- or heterodimerize. When two plasmids encoding these constructs are expressed in *Escherichia coli*, the two interacting partners recognize each other, allowing the two halves of mDHFR to come into close contact, thus restoring its enzymatic activity. The *E. coli* DHFR is inhibited by the antibiotic trimethoprim (TMP), whereas the murine DHFR does not show inhibition. Thus, functional mDHFR confers on *E. coli* the ability to grow on minimal medium in the presence of TMP. This system had been applied successfully to select for leucine-zipper domains with optimized binding properties in a bait-*versus*-library and also in a library-*versus*-library selection (Arndt *et al.*, 2000; Pelletier *et al.*, 1999). It has been shown to work rapidly and reliably, due to the negligible background of false positives. One important advantage of this system is that, unlike in phage display, the antigens do not have to be expressed and purified, as only the DNA of the antigen has to be cloned to provide the DHFR-fusions *in situ*.

In the present study, we addressed the question of whether antibodies in the single-chain Fv (scFv) format can be used in the PCA system. One possible obstacle was the fact that the mDHFR complementation takes place in the reducing environment of the cytoplasm of *E. coli.* There, the intradomain disulfide bonds of the scFvs will not form. As these disulfide bonds contribute about 4-5 kcal mol^{-1} to the overall stability of the scFv, antibodies are strongly destabilized in their reduced form (Frisch *et al.*, 1996). In fact, several scFv antibodies have been reported to be quite sensitive towards the absence of their stabilizing disulfide bonds (Glockshuber *et al.*, 1992; Proba *et al.*, 1995). However, some intrinsically stable scFv antibodies show tolerance against the loss of their disulfide bonds, and they can be expressed functionally in the *E. coli* cytoplasm (Chen *et al.*, 1994; Proba *et al.*, 1998). Several scFv antibodies have been tested for activity in a yeast two-hybrid assay, and indeed some showed activity in the cytoplasm of *Saccharomyces cerevisiae* (Cattaneo & Biocca, 1999; De Jaeger *et al.*, 2000; Visintin *et al.*, 1999). A stability-engineered anti-GCN4 scFv was expressed in the cytoplasm of *S. cerevisiae*. Functionality was demonstrated by the fact that the activity of β-galactosidase, expressed from a GCN4-dependent reporter gene, was suppressed by the action of the antibody to about 18 % of wild-type activity, resulting in the generation of a functional knock-out (Wörn *et al.*, 2000). These results clearly demonstrate the potential that at least some stable and well folding antibodies have for being used in cytoplasmic selection procedures, and the generation of diverse libraries with all members being this stable is within reach.

The above-mentioned engineered anti-GCN4 scFv, already shown to be functional in the yeast cytoplasm, was used to check the suitability of scFv antibodies for the PCA system. This anti-GCN4 scFv recognizes a random-coil conformation of the dimerization domain of the GCN4 leucine zipper motif (Berger *et al.*, 1999; Hanes *et al.*, 1998). This scFv was fused to either of the two mDHFR

domains. Also, the GCN4 leucine zipper was fused to either of the two mDHFR domains. In addition to the wild-type GCN4 leucine zipper, a mutant zipper (named 7P14P) was used. This 7P14P mutant carries two additional proline residues to disrupt the helical structure of the zipper, these mutations therefore prevent its coiled-coil-mediated homodimerization (Berger *et al.*, 1999; Leder *et al.*, 1995). The use of this mutant should simplify the process of antibody/antigen recognition, because there is only an equilibrium between free and antibody-bound antigen. In contrast, the wild-type GCN4 homodimerizes, and thus antibody/antigen binding has to compete with the homodimerization process.

We optimized the fusions of the antibody and the antigen by variation of the linker length between the antigen or antibody and its DHFR domain fusion partner and by swapping the DHFR domains. For this purpose, four different linker lengths between each DHFR fragment and its fusion partner were tested in order to find an optimum between possible geometrical restrictions and an unnecessary reduction in effective local concentration through the use of excessively long linkers. We used linkers of the sequence $(Gly_4Ser)_n$, with $n = 0$, 2, 3 or 4, which are flanked by some additional amino acid residues (Figure 2).

Each GCN4-mDHFR-I fusion construct bearing one of the different linkers was cotransformed in *E. coli* cells in a pairwise manner with constructs of the different anti-GCN4-mDHFR-II fusions (Figure 2). The transformed bacteria were plated on M9 minimal agar in the presence of TMP, thereby allowing cells to grow only as a result of functional complementation of the two mDHFR fragments. The plasmids encoding GCN4-mDHFR-II with all different linkers were similarly cotransformed with all anti-GCN4-mDHFR-I constructs. Thus, all linker lengths were tested *versus* each other. The efficiency of restoring DHFR function with each set was quite asymmetric: the scFv-mDHFR-II fusions, together with the GCN4-mDHFR-I fusions (Figure 2(a)), give rise to far fewer colonies than the opposite pairing (Figure 2(b)). This behavior can be observed for the wild-type GCN4 zipper as well as for the 7P14P mutant zipper (compare Figure 2(a) and (c) with (b) and (d)). These results indicate that fusion of the scFv to fragment-II of mDHFR has deleterious effects on the complementation behavior of the fragments. The exact reason for this is not known, but one could speculate that the scFv, which itself is sensitive to aggregation, negatively influences the stability and solubility of the mDHFR-II fragment more than that of mDHFR-I. Therefore, the concentration of active mDHFR-II is decreased in the cell, which diminishes the DHFR complementation. To test this, Western blot analyses were performed for all constructs (data not shown). In all cases, the fusion proteins were found mostly in the insoluble lysate fraction, and only a small part was in the soluble fraction. However, no significantly different expression patterns could be detected for any of these fusion proteins by Western blot.

As several pairs of linkers resulted in equally high numbers of colonies when using the 7P14P mutant zipper in the cotransformation experiments (Figure 2(d)), we chose the pair of linkers that proved to be the best when using the wild-type zipper fusions (Figure 2(b)). This was the anti-GCN4-mDHFR-I construct, where a $(Gly_4Ser)_2$ linker has been added to the existing linker to result in a linker of 21 amino acid residues in total. In the GCN4-mDHFR-II fusion, the $(Gly_4Ser)_4$ linker sequence was added to give a linker length of 32 amino acid residues (Figure 2(b)). In this case, we obtained over 18 % of the colony number of the homodimerizing zipper domains of GCN4 and, in the case of the 7P14P mutant, even values between 50 % and 90 % were obtained. This result shows that under these conditions antibodies can be selected nearly as effectively as interacting helical peptides.

In the next step, we wanted to test whether other antigen/antibody pairs would be functional in this assay and whether the antibodies still specifically recognize their respective epitopes under the conditions of the cytoplasm. Therefore, we chose several candidate scFv antibodies directed against peptides and whole proteins. The anti-HAG antibody 17/9 used here (Schulze-Gahmen *et al.*, 1993; Krebber *et al.*, 1995), recognizes a peptide of six residues from the haemagglutinin protein of influenza virus and was evolved for enhanced stability, using ribosome display, in the presence of the disulfide reductant DTT (Jermutus *et al.*, 2001). We also investigated antibodies that recognize folded proteins as their antigen. We used four different scFvs that were obtained by phage panning of the HuCAL-library (Knappik *et al.*, 2000; B. Krebs *et al.*, unpublished results) *versus* the peptidyl-prolyl *cis/trans* isomerase FkpA from *E. coli* (Missiakas *et al.*, 1996; Bothmann & Plückthun, 2000; Ramm & Plückthun, 2000). The anti-FkpA scFvs will be described elsewhere (K. Blank, B. Krebs *et al.*, unpublished results). All five antibodies and their two antigens were cloned into the vectors and fused to the respective mDHFR fragments, using the above-defined optimal linker pairs. While the possibility cannot be excluded that a different optimal length exists for every antigen/antibody pair, it should be noted that several of the longer linkers seemed to yield a comparable level of complementation. We therefore believe that, with these long linkers, many antibody/antigen pairs can be tested.

For the complementation assays, each antibody-containing plasmid was cotransformed with a plasmid encoding its cognate antigen and plated under selective conditions with expression of the fusions induced. The resulting colony numbers are shown in Table 1. The results show that the GCN4/anti-GCN4 system and the HAG/anti-HAG system gives rise to a large number of clones (about 10^8 per μg DNA) when cotransformed with the

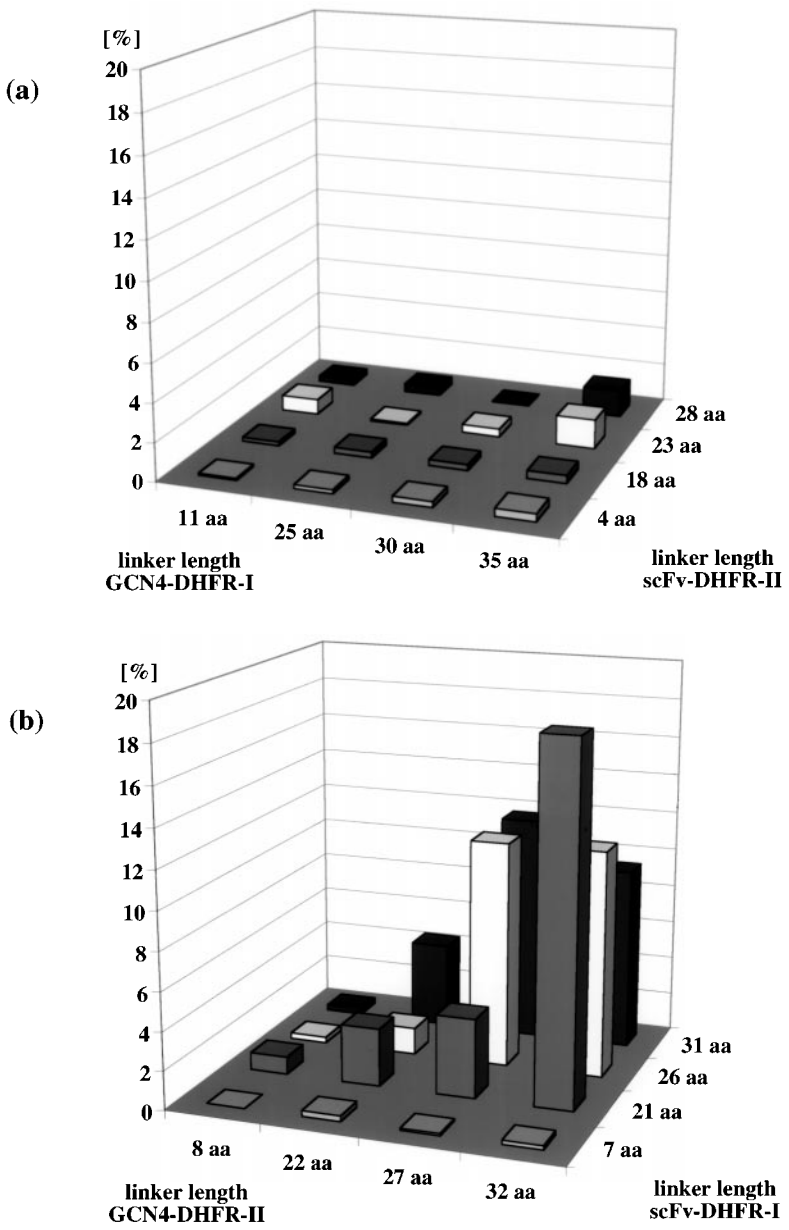

Figure 2 (*legend opposite*)

respective cognate partner, and therefore support the reassembly of mDHFR. As a negative control, the same experiment was performed when the cells were plated on M9 agar plates without IPTG, conditions under which no fusion proteins were expressed. Here, the number of clones dropped drastically, by six to seven orders of magnitude. This clearly shows that the expression of the fusion constructs is responsible for the ability to grow on minimal medium in the presence of the antibiotic TMP. In the case of the anti-FkpA, only two of the four scFvs, named 9B3 and 7B2, gave rise to a significant number of colonies. The other clones, 6B1 and 11B4, did not show a number of colonies significantly over the background level. Therefore, these two antibodies are not included in Table 1.

There are several possible reasons why 6B1 and 11B4 do not show functionality in this complementation assay. These antibodies might recognize an epitope close to the termini of the antigen. As all constructs contained a His-tag on the N terminus and the mDHFR fusion protein on its C terminus, the termini might be invisible for the antibodies, which were obtained by phage panning against the wild-type protein. It might be possible that these two antibodies do not adopt a folded conformation in the cytoplasm, due to the lack of stabilizing disulfide bridges and a lower intrinsic stability than the other scFv constructs tested. Also, geometric

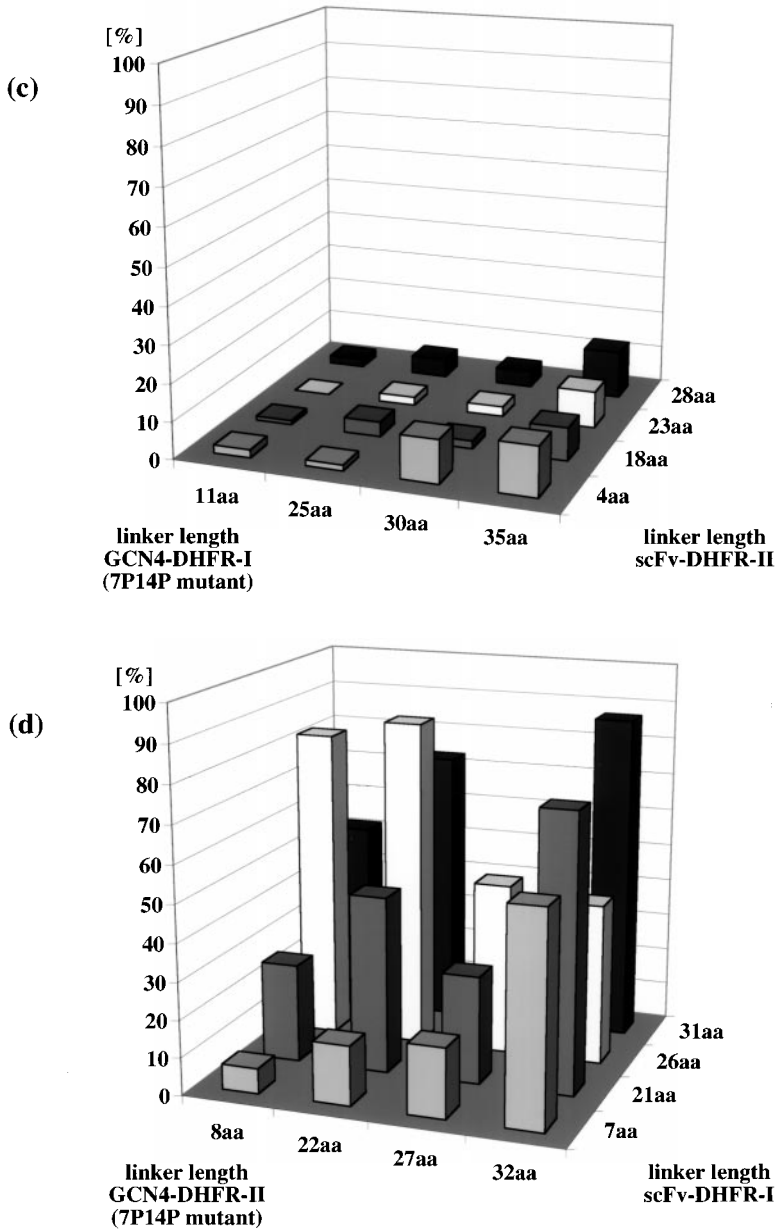

Figure 2. Influence of the linker length between the fusion partners on the efficiency of DHFR complementation. The GCN4 leucine zipper domain and the anti-GCN4 scFv antibody were genetically connected either to fragment I or fragment II of mDHFR. (a) Plasmids encoding the anti-GCN4-scFv fused to fragment II of mDHFR were cotransformed with plasmids encoding the GCN4 zipper domain fused to fragment I of mDHFR. Numbers represent the percentage of colonies obtained with one particular pair of plasmids in comparison to the positive control (GCN4 leucine zipper domains fused to either of the two mDHFR fragments as described (Pelletier *et al.*, 1998). (b) The anti-GCN4-scFv was fused to fragment I of mDHFR, and the GCN4 zipper domain fused to fragment II of mDHFR: (c) and (d) correspond to (a) and (b), except that the GCN4 zipper domain contained two proline residues (7P14P) to disrupt the helical structure, resulting in a random coil, preventing coiled-coil-mediated homodimerization (Leder *et al.*, 1995). The anti-GCN4-scFv is derived from work by Wörn *et al.* (2000). The GCN4-mDHFR-I and II constructs are from Pelletier *et al.* (1998) and already contain a linker of 11 or eight amino acid residues, respectively, to which the different Gly_4Ser linkers were added. The antibody-mDHFR-I and II fusions contain another seven or four amino acid residues, respectively, in the linker region for cloning purposes. These linker components thus add up to the numbers on the axes of the Figure. Cotransformation of plasmids was performed by electroporation of 10 ng of each plasmid into BL21/pREP4 cells (Qiagen). Transformed cells were plated on M9 minimal agar plates in the presence of 2 $\mu g\ ml^{-1}$ trimethoprim (TMP), 1 mM IPTG, 50 $\mu g\ ml^{-1}$ kanamycin and 100 $\mu g\ ml^{-1}$ ampicillin. Numbers of colonies were counted after four days of growth at 25 °C. The same experiment was performed in the absence of IPTG, to show that TMP-resistance occured only *via* expression of the fusion partners and no colonies were observed (data not shown).

Table 1. Number of colonies obtained after cotransformation of the scFv-mDHFR-I fusions with the plasmids encoding antigen-mDHFR-II fusions

	Antigen-DHFR-II fusions[a]		
scFv-DHFR-I fusions	Specific antigen[b]	Antigen pool containing the specific antigen[c]	Antigen pool without the specific antigen[d]
Anti-GCN4	3.3×10^8	3.3×10^7	20 ± 13
Anti-FkpA (7B2)	5.6×10^7	1.1×10^7	20 ± 7
Anti-FkpA (9B3)	3.0×10^7	1.1×10^7	0 ± 0
Anti-HAG	1.3×10^8	2.6×10^7	13 ± 13

Cotransformation of plasmids was performed as described for Figure 2, except that 100 ng instead of 10 ng of each plasmid was transformed in a single experiment. Growth conditions were as for Figure 2. Numbers given in the Table are extrapolated to 1 μg of DNA per transformation. As a negative control, the same experiment was performed in the absence of IPTG (data not shown). Here, the number of colonies was about the same as in the non-cognate control (last column). All experiments were carried out in duplicate.

[a] Number of colonies per μg DNA.
[b] Cotransformation with the cognate plasmid only.
[c] Cotransformation with a pool of plasmids, containing all three different antigen-DHFR-II fusions.
[d] Cotransformation with a pool of plasmids, containing only the two non-cognate antigen-DHFR-II fusions.

constraints cannot be excluded, since the FkpA protein consists of 244 amino acid residues, and even long linkers would not allow the scFv to reach every epitope of the antigen. As in the case of the anti-GCN4-fusion, Western blot analysis of all four anti-FkpA-mDHFR constructs showed that the major part of the protein was found in the insoluble lysate fraction, but no significant differences were observed between the antibodies tested. The question of whether there is a limiting threshold in affinity can be addressed only after a statistically relevant number of binders is available from selection experiments.

The next issue we wanted to address was the specificity of the antigen/antibody recognition under the conditions present in the cytoplasm. Therefore, we mixed all plasmids expressing an antibody fusion in two separate experiments, either with a pool of all plasmids containing the antigen fusions, or with a pool of those plasmids containing all antigens except their respective cognate partner (Table 1). The data show that the pool containing the cognate antigen yielded roughly the same number of colonies as when only the cognate plasmids were used (>10^7 clones per μg DNA transformed). In the case of the pool without the cognate pairing, only the number of clones was observed that corresponds to the background (approximately ten clones per μg DNA transformed). These results demonstrate that all antibodies used in this study interact either specifically with their partner or show no reactivity at all. This is an important observation, since it suggests that large-scale screening and selection of interacting proteins should be feasible with this system.

In order to prove that all clones really did arise due to specific interactions, we checked ten to 15 of the clones that appeared in each of the experiments with pooled antigen *versus* single antibody plasmids for the presence of the correct pair of plasmids. For this purpose, these colonies were picked and put into a PCR reaction mix that contained one pair of oligonucleotide primers that would anneal in both plasmids just outside of the open reading frame of the fusions. Therefore, two different products would be obtained, one for each plasmid (see the legend to Table 2). Since the DNA sequences for the different antigens and antibodies

Table 2. Frequency of observed antigen/antibody pairs in a library-*versus*-library experiment

	GCN4	FkpA	HAG
Anti-GCN4-scFv	15	0	0
Anti-FkpA-scFv	0	16	0
Anti-HAG-scFv	0	0	17

Plasmids encoding three different scFv-DHFR-I and three different antigen-DHFR-II fusions were mixed and transformed into *E. coli* in a single experiment. Colonies that were obtained on minimal agar in the presence of IPTG and TMP (same experimental conditions as for Table 1) were analyzed by colony-PCR as described below. Numbers represent the occurrence of particular pairs of antigen and antibody. Note that each cognate pairing was found at similar frequencies because cells showed different growth rates, and colonies appearing on the second, third and fourth day were picked and analyzed. Colonies that appeared on the second day harbored mostly the FkpA/anti-FkpA (15/16) and only once the GCN4/anti-GCN4 pairing. The next day mainly GCN4/anti-GCN4 pairs became visible (13/16) and (3/16) showed the HAG/anti-HAG pairs. On the fourth day the HAG/anti-HAG pairing was observed 14 times and the GCN4/anti-GCN4 pair only twice.

PCR analysis of colonies obtained after cotransformation experiments was performed as follows. One pair of oligonucleotide primers was used, which annealed outside of the open reading frame (ORF) of the fusion constructs. Since identical plasmids were used in each case (Pelletier *et al.*, 1998, 1999), the size of the PCR product depends only on the size of its ORF. Two bands were expected in agarose gel electrophoresis for each experiment, one for the antigen and one for the antibody. Each antibody contained different diagnostic restriction sites. By digestion with different enzymes, the presence or absence of each antibody could be determined unequivocally.

all contain different diagnostic restriction sites, the presence of the cognate antigen and antibody DNA could be determined by simple digestion of the resulting PCR products. In all cases investigated, we observed DNA products only from the specific antigen/antibody pairs.

In a final set of experiments, three plasmids encoding the anti-FkpA, anti-GCN4 and anti-HAG scFvs (fused to DHFR-I) and the three respective antigen fusions to DHFR-II were mixed. This mixture of six plasmids, all having ampicillin resistance, can give rise to six different single transformants and 15 different double transformants, of which nine are antibody/antigen pairs and only three are cognate pairs. Using the PCR diagnostics described above, in 48 colonies grown under selective conditions in minimal medium, exclusively cognate pairs were obtained (Table 2). All three cognate pairs were found, each among colonies of a particular size (see below). This experiment thus shows the first library-*versus*-library demonstration with antibodies and antigens.

One interesting observation was that the growth-rate of the *E. coli* cells was dependent on the different antigen/antibody pairs that it contained. Colonies that appeared after two days of growth at 25 °C harbored exclusively the FkpA/anti-FkpA pairs. One day later the GCN4/anti-GCN4 pairing could be observed, and the HAG/anti-HAG pair became visible only on the fourth day. Whilst this may reflect the solubility and folding properties of the DHFR fusions, and possibly the antibody affinities, it is conceivable that the antigen FkpA, recently found to have some chaperone activity itself (Bothmann & Plückthun, 2000; Ramm & Plückthun, 2000), would also improve the folding yield of scFv-fusions in the cytoplasm of *E. coli*. Undoubtedly, these observations require further investigations. These results do show clearly, however, that only a specific interaction of antibody and antigen leads to a functional complementation of the mDHFR system.

Therefore, this system seems to be suitable for the selection of antibodies and for the determination of antigen epitopes. Since the antibodies need to be functional under reducing conditions, they are required to have stable frameworks. We are currently optimizing libraries for this purpose. We believe that the protein fragment complementation assay is the simplest conceivable way to select antibodies from a library. It requires only transformation of plasmids, functional expression of the fusion proteins, and analysis of the grown bacterial cells. Its main attraction is that it might allow library-*versus*-library applications on a large scale; for example, the selection of antibodies against a whole cDNA library, a protein domain library or a peptide library. It has been shown to work with both peptides and protein antigens, even though it does require that at least some of the antigen fusion is soluble and withstands proteolytic degradation. For reasons of library size and simplicity, the selection in *E. coli* is preferred whenever possible. However, one limitation of this system is that the usage of *E. coli* prevents the analysis of post-translationally modified proteins, and further screens in other cells may be required.

In summary, we believe that the remarkable ''signal-to-noise'' ratio of seven orders of magnitude (number of specific *versus* unspecific colonies) makes this system very interesting for high-throughput antibody generation in functional genomics. Furthermore, no antigen needs to be purified and immobilized. Finally, the astonishing simplicity of merely growing *E. coli* on minimal medium as a means for identification of cognate pairs should allow an easy implementation of this technology in every research lab and also the upscaling for functional genomics.

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