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Direct Selection of Antibodies from Complex Libraries with the Protein Fragment Complementation Assay

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The aim of the present study was to develop the protein fragment complementation assay (PCA) for the intracellular selection of specific binding molecules from the fully synthetic HuCAL® antibody library. Here, we describe the first successful selections of specific antibodies by PCA, and we discuss the opportunities and limitations of this approach. First, we enriched an antibody specific for the capsid protein D of bacteriophage lambda (gpD) by ten successive rounds of competitive liquid culture selection. In an independent approach, we selected a specific antibody for the c-Jun N-terminal kinase 2 (JNK2) in a single-step selection setup. In order to obtain specific antibodies in only a single PCA selection round, the selection system was thoroughly investigated and several strategies to reduce the amount of false positives were evaluated. When expressed in the cytoplasm of *Escherichia coli*, the PCA-selected scFv antibody fragments could be purified as soluble and monomeric proteins. Denaturant-induced unfolding experiments showed that both antibody fragments are stable molecules, even when the disulfide bonds are reduced. Furthermore, antigen-specificity of the PCA-selected antibody fragments is demonstrated by *in vivo* and *in vitro* experiments. As antigen binding is retained regardless of the antibody redox state, both PCA-selected antibody fragments can tolerate the loss of disulfide bridge formation. Our results illustrate that it is possible to select well-expressed, stable, antigen-specific, and intracellular functional antibodies by PCA directly.

Keywords: intrabodies; protein fragment complementation assay; HuCAL® antibody library; *in vivo* selection; cytoplasmic expression

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Introduction

An extensive list of DNA sequences, coding for proteins with unknown functions, is being generated by the ongoing worldwide genome sequencing programs.[1] Elucidating the functions of these proteins will be one of the most important challenges in the years to come.[2] Therefore, one important task of biotechnology is to generate specific binding molecules against a wide variety of targets with reasonable resources. Although a variety of selection systems have proven success in generating such binding molecules and are thus commonly used, it is unlikely that they will provide the required throughput without additional components and development. In most display techniques the purification and immobilization of the target molecules has to be worked out individually, and binders against every target have to be selected in separate experiments. Consequently, there is great demand for a method that would allow

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Abbreviations used: BSA, bovine serum albumin; CDR, complementary-determining region; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidine hydrochloride; gpD, capsid protein D of bacteriophage lambda; HuCAL, human combinatorial antibody library (MorphoSys AG, Munich); IPTG, isopropyl-β-D-thiogalactopyranoside; JNK2, c-Jun N-terminal kinase 2; mDHFR, murine dihydrofolate reductase; PCA, protein fragment complementation assay; RU, resonance unit; scFv, single-chain antibody fragment consisting of the variable domains of the heavy (V_H) and light chain (V_L) connected by a peptide linker; SPR, surface plasmon resonance; TBS, Tris-buffered saline; TMP, trimethoprim; wt, wild-type.

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substantially faster selection of binding molecules.[3,4] Since the protein fragment complementation assay (PCA) might have the potential to become a robust but very simple selection technology, and even allows parallel selection against several targets in the same experiment, the aim of the present study was to test and develop PCA for the intracellular selection of specific binding molecules from two different single-chain antibody libraries. The use of PCA to select from naïve protein libraries directly has not been described before.

The PCA strategy described here is based on the genetic dissection of the murine enzyme dihydrofolate reductase (mDHFR).[5–7] In this approach, two interacting partners (here, antibody and antigen) are genetically fused to the two halves of the divided mDHFR. When *Escherichia coli* is co-transformed with both plasmids, the fusion partners can dimerize with each other and thereby reassemble the dissected enzyme from its individual fragments. Since bacterial DHFR is inhibited specifically through the antibiotic trimethoprim and thus cell division cannot occur, the reassembled mDHFR restores the biosynthetic reactions required for bacterial propagation. Antibody–antigen interaction is thereby linked directly to bacterial survival and is detectable simply by colony formation. For carrying out PCA selections, only the genes of the target and of the binding molecule have to be available. Therefore, a very rapid identification from libraries and thus "generation" of specific binding molecules is conceivable, and the work of purifying and immobilizing the antigen of interest is not required, at least before more detailed investigations of the binders are carried out. In summary, PCA requires only transformation of plasmids, functional expression of the fusion proteins, and analysis of bacterial cells. Consequently, we believe that this technology may offer great potential in terms of speed, simplicity, and future automation.

In order to test whether the PCA system would be suitable for use with antibodies, several scFv fragments were tested in previous model experiments.[8] In these experiments, all evaluated antibodies were able to reassemble the separated mDHFR domains into a functional enzyme through specific binding to its cognate target. In addition, every antibody that had been used in this study interacted either specifically with its antigen or showed no reactivity at all. This was an important observation, since it suggests that selection of antibodies should be feasible with PCA.

Nevertheless, utilization of recombinant antibody fragments in the reducing environment of the cytoplasm regularly leads to folding and stability problems due to restricted formation of their intrachain disulfide bond.[9] Consequently, low levels of expression of soluble protein and limited half-lives of antibody domains, which are either aggregating or being degraded proteolytically, are usually observed when expressed in the cytoplasm. Furthermore, incorrectly folded molecules may engage in undesired and unspecific interactions and thereby greatly complicate the selection process.

To overcome the problem of incomplete antibody folding in the bacterial cytoplasm, several approaches had been suggested: either the antibodies were expressed as fusions to a very soluble protein,[10] which leads to enhanced solubility of the fused antibody domains, albeit not necessarily to a completion of the folding process, or the antibodies were produced in modified *E. coli* strains that allow more efficient oxidation of the cytoplasmically expressed antibodies.[11–13] In alternative strategies, the optimization of individual antibodies was carried out in order to enhance the thermodynamic stability or the solubility and expression level of a particular molecule.[14–19] Such subsets of antibodies have been expressed in the bacterial cytoplasm in a stable and functional form, even though some residual aggregation or soluble aggregate formation may well occur. Additionally, the stability-engineered antibodies were used as frameworks to which other specificities have been grafted.[20,21]

Even though such single-framework antibody libraries appear suitable for direct intracellular selection, they have been usually applied to *in vitro* (most often phage display) selections, rather than in intracellular selections from the complex library.[22,23] Instead, the intracellular performance of the selected antibodies was usually evaluated in subsequent experiments, separate from the selection itself. Since it is not ensured that the phage-selected antibodies, which contain disulfides during the selection, bind their antigen also in the reducing cytoplasm of a cell, it has become a routine procedure to perform a yeast two-hybrid screen, subsequent to an initial phage display selection round.[24,25] This two-step selection strategy allows the isolation of antibodies based on their *in vivo* binding activity, and by transforming only a small pool of phage-selected binders, the problem of low transformation efficiency of yeast is overcome. Nevertheless, this two-step procedure remains laborious, and the yeast two-hybrid method is used only for an evaluation of binders, rather than a selection strategy itself. In contrast, the high transformation efficiency of *E. coli* should allow the direct selection of antibodies in the bacterial cytoplasm. Thus, utilization of PCA would allow the combination of both antibody selection and antibody evaluation in only one process and might, therefore, greatly improve the throughput of selection.

Although it has been possible to increase the stability of individual antibodies, it might be more promising to apply designed antibody libraries in which a high proportion of molecules remain functional under reducing conditions of the bacterial cytoplasm. For this purpose, we assembled two antibody libraries in the scFv format. In order to start the PCA selections from well-expressed antibody frameworks, suitable for cytoplasmic expression, we utilized a restricted group of very stable HuCAL® (Human Combinatorial Antibody Library, MorphoSys AG, Munich) master framework combinations. Since the stability, the expression yield

and the aggregation behavior of all human variable antibody domains has been investigated recently in a comprehensive study,[26] only the domains possessing the most favorable properties for an intracellular application were included in our scFv antibody library construction. Fortunately, the modular design of the HuCAL® antibody library[27] allowed us to combine the most stable heavy chain (V_H3) with all seven types of light chain domains in our first scFv antibody library. In this library, complementary-determining regions (CDR) CDR-H3 and CDR-L3 are diversified as described,[27] while CDR1 and CDR2 of both heavy and light chain are consensus sequences according to the subgroup. In the second approach, the constructed scFv antibody library resulted from a combination of both the most stable heavy chain domain (V_H3) with the most stable light chain ($V_\kappa3$) domain. In this case, the complexity of the latter, so-called "single-framework" antibody library resulted from diversification of all six CDR cassettes of both the heavy chain domains and the light chain domains (S. Urlinger, C. Rothe *et al.*, Morphosys A.G., unpublished results). Both types of antibody libraries were constructed with a complexity of more than 10^9 transformants. The performance of both antibody libraries in the reducing environment of the bacterial cytoplasm has been investigated thoroughly, and the direct selection of functional intracellular antibodies from the assembled libraries is reported. We critically discuss the opportunities and current limitations of direct PCA selection.

Results

Construction of the first antibody library

The reducing environment of the cytoplasm regularly leads to folding and stability problems with antibody fragments, due to the restricted formation of their intra-chain disulfide bonds. To overcome this limitation, we decided to assemble two antibody libraries in scFv format from selected HuCAL® frameworks possessing favorable properties. In the first library, the most stable heavy chain subtype (V_H3) was combined with all seven types of light chain domains ($V_\kappa1$–$V_\kappa4$, $V_\lambda1$–$V_\lambda3$)[27] to guarantee sufficient antigen-binding diversity. The diversification of the CDR3 cassettes of both the heavy chain domains and the light chain domains constituted the main diversity of the antibody library.[27] The assembled antibody library possesses a final diversity of 4.5×10^9 transformants, which corresponds to an approximately tenfold over-sampling of the theoretical library size, based on the number of input clones.

Initial selection experiment with serial transfers

After construction of the first antibody library, the library-mDHFRI fusion proteins were applied in a selection for binding to the capsid protein D of bacteriophage lambda (gpD).[28] This particular antigen was chosen because of its favorable expression behavior. gpD is a small protein (11.4 kDa) that does not contain cysteine and shows high levels of soluble expression in the cytoplasm of *E. coli*.

The selection experiment started with a co-transformation of the plasmids encoding the antibody library fused to mDHFRI and the antigen (gpD)-mDHFRII fusion protein. Plating on minimal medium was used to determine the bacterial survival rate under selective conditions. Unexpectedly, we observed a very high survival rate of this particular test-transformation (approximately one million colonies), and thus decided to perform serial transfers in liquid culture for selecting the best binders against gpD. We reasoned that the performance in consecutive rounds of liquid culture selection should lead to an enrichment of the binding molecules that form the most stable interaction. Since a more stable interaction might lead to a larger amount of reassembled active mDHFR, the growth rate of the particular *E. coli* cell might be increased as well. Therefore, the corresponding antibody should become enriched specifically during performance of multiple selection cycles.

For carrying out this selection, *E. coli* was co-transformed again with the antibody library and the antigen construct, and subsequently transferred to a flask containing liquid selection medium. After ten consecutive selection rounds, the selection was analyzed, although we still observed accelerated bacterial growth (Figure 1). A diluted sample of the last selection round was transferred to a selection plate. From this selection plate, the antibody-encoding plasmids of 30 independent colonies were isolated and sequenced. No antibody-encoding plasmid containing a frameshift or stop codon in the antibody-mDHFRI sequence was found. Additionally, no recombination between the antigen-encod-

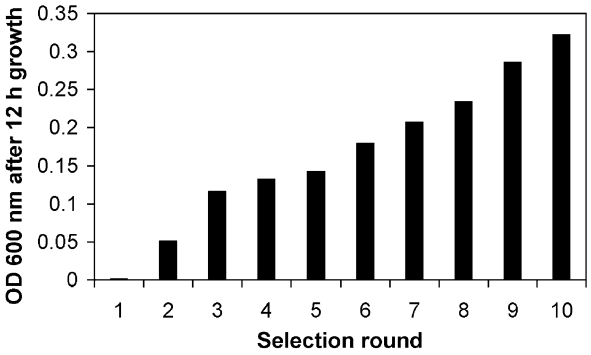

Figure 1. Monitoring bacterial growth of ten successive rounds of liquid culture selection against gpD. After co-transformation of the gpD-mDHFRII fusion protein with the antibody library-mDHFRI fusion protein encoding plasmids, the bacteria were transferred to a shaker flask containing liquid selection medium. Each selection round was inoculated to the same absorbance ($A_{600\ nm}=0.001$) from an aliquot of the previous selection round. All ten selection rounds were incubated for 12 h each. The accelerated bacterial growth, visible for each 12 h time-window, should represent an enrichment of antibodies inducing higher enzymatic mDHFR activity.

ing and the antibody-encoding plasmids was detected. However, in two out of 30 constructs investigated, a deletion of the antibody genes was found. Yet, these observed occasional deletions are most probably not present in the cell but rather result from the isolation strategy of the antibody-encoding plasmids (see Materials and Methods). None of the sequenced antibodies was found multiple times.

Since the growth rate was still increasing from round to round (Figure 1), liquid culture selection was continued. After another five rounds (a total of 15 serial transfers of the *E. coli* culture), the growth rate did reach a plateau value. Sequencing of some of the clones from the 15th selection round showed that the antibody diversity was only slightly reduced (of 15 antibodies, 12 were different), when compared to the diversity of the tenth selection round (of 30 antibodies, all were different). This result shows that a diverse number of antibodies could meet the selection criteria, even though only one specifically interacting pair was found (see the next section).

In order to simplify the specificity verification of PCA-selected antibodies, we expressed the selected antibodies in the mDHFRI fusion format directly and performed an antigen enzyme-linked immunosorbent assay (ELISA) with crude extracts of the antibody expression cultures. Finally, out of the 30 randomly chosen antibody fusions from the tenth selection round, one antibody specifically binding the antigen gpD (antibody D10) was identified in our ELISA setup (data not shown). This antibody was retained after the 15th serial transfer.

D10 represents thus the first specific antibody that has been selected successfully by PCA from a naive antibody library. Although its functionality was shown in the reducing intracellular environment, it contained no mutation in its constituent V_H3-$V_\lambda3$ antibody domains. This implies that the antibody D10 retains functionality in the reducing environment of the bacterial cytoplasm, even though its intra-chain disulfide bonds are not formed. The further characterization of D10 is described below.

Strategies to reduce unspecific bacterial growth

From the large number of antibodies that had become enriched in the initial PCA selection (all correct in sequence), it became clear that in most cases unspecific antibody–antigen interaction events led to bacterial growth. Unspecific bacterial growth did not result from genetic instability of the PCA constructs. In order to reduce unspecific mDHFR activity, the influence of several parameters on bacterial growth under selective conditions was evaluated.

Utilization of a mDHFRII point mutant

In this co-transformation experiment, two different types of mDHFRII fragments (fusion partner of the antigen) were utilized: the wild-type (wt) mDHFRII fragment, which has been used in our initial PCA selection against gpD, and an mDHFRII variant containing the I114A point mutation.[5] By utilizing this latter, weakly associating mDHFRII fragment, the stringency of selection should be increased. We reasoned that only binding molecules that form a rather stable interaction would compensate the reduced ability of the mDHFRII I114A fragments to associate into the active enzyme. In order to verify the effect of the mDHFRII mutant, the gpD-specific antibody D10 and ten gpD unspecific antibodies resulting from the initial PCA selection against gpD were co-transformed with both the wt gpD-mDHFRII fusion protein and the gpD-mDHFRII I114A mutant construct. For all of the chosen antibodies, bacterial growth was reduced significantly if the mDHFRII I114A mutant was co-transformed (Figure 2(a)). However, since this effect was identical for both the gpD specific antibody D10 and all unspecific antibodies tested, utilization of the mDHFRII I114A mutant does not seem to improve the PCA selection

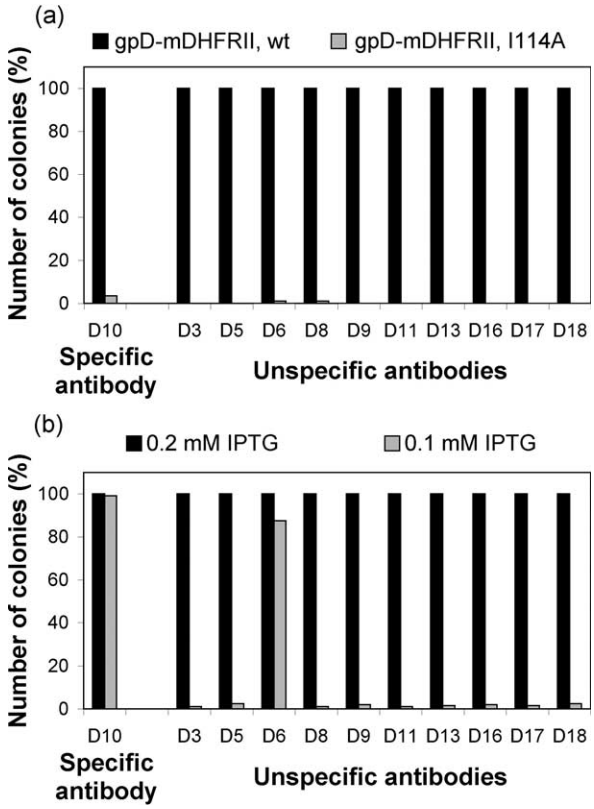

Figure 2. Test of two strategies to reduce unspecific bacterial growth. (a) Influence of the interaction strength of mDHFRI and mDHFRII on specific and unspecific antibody–antigen interaction pairs. Bacterial growth resulting from co-transformations utilizing the wild-type (wt) gpD-mDHFRII fusion protein (black bars) was set to 100% and compared to bacterial growth resulting from co-transformations of the mDHFRII I114A mutant (grey bars). (b) Influence of induction conditions on specific and unspecific antibody–antigen interaction pairs. Bacterial growth resulting from induction with 0.2 mM IPTG was set to 100% (black bars) and compared to bacterial growth resulting from induction with only 0.1 mM IPTG (grey bars).

system in rewarding cognate interactions over non-cognate ones.

Influence of reduced induction strength

The initial PCA selection against gpD was performed at a high induction level (1 mM isopropyl-β-D-thiogalactopyranoside (IPTG)) to induce the expression of the mDHFR fusion proteins. As this high induction strength may have induced the fusion proteins to aggregate, a simple reduction of the expression level might reduce the proportion of misfolded antibodies, and unspecific bacterial growth might become more limited. To investigate the effect of reduced induction strength, we co-transformed the gpD-specific antibody D10 and ten gpD-unspecific antibodies with the wt gpD-mDHFRII antigen construct and incubated the transformed cells on selection plates containing different concentrations of IPTG. Reduction of the concentration of IPTG from 1 mM to 0.2 mM had almost no effect on all investigated interaction pairs. Neither the number of colonies nor their size was reduced. However, a completely different result was observed upon a further decrease of the concentration of IPTG to 0.1 mM IPTG (Figure 2(b)). Under these conditions, almost no viable bacterial colonies were detected on the selection plates for nine out of ten unspecific antibodies. In contrast, there was no influence on the specific antibody–antigen pair that had been selected by PCA before. Thus, we concluded that the reduction of induction strength might be a promising strategy to refine the PCA selection system.

Construction of the second antibody library

Sequence analysis of unselected library members had shown an unbiased distribution of all different light chain subgroups (data not shown). In contrast, sequencing of the antibody fusions, which had been enriched in our initial PCA selection against gpD, revealed that approximately 90% of the unspecific antibodies were comprised of the light chain domains $V_\lambda 2$ and $V_\lambda 3$, which when unpaired are least stable.[26] This led us speculate that these antibodies might not be able to fold properly in the bacterial cytoplasm, and might therefore lead to mDHFR reconstitution through unspecific interactions with the antigen and/or the DHFR fragment. To reduce bacterial growth that is not dependent on cognate antibody–antigen interactions, the design of the scFv antibody library was then revised, focusing on a more restricted group of stable antibody frameworks. Thus, in a second approach, the modular design of the HuCAL® Gold antibody library was used to combine the most stable heavy chain (V_H3) with only the most stable light chain ($V_\kappa 3$) domain. The binding diversity of this new single-framework antibody library resulted from a diversification of all six CDR cassettes. Construction of the particular scFv antibody library finally yielded a diversity of 3.8×10^9 transformants, which represents, in contrast to our first antibody library, no over-sampling of the theoretical library size.

Selection experiments under improved conditions

In order to verify whether the proposed improvements (library and reduced induction strength) lead to a PCA selection of specific clones more rapidly, a new selection was carried out. In contrast to our first selection experiment, two antigens were utilized in this second series of selections. Besides using gpD, again for reasons of high soluble expression, we additionally used the c-Jun N-terminal kinase-2 (JNK2)[29] as antigen, a protein that has attracted considerable scientific interest as a potential drug target.[30] JNK2 is approximately five times larger than gpD (48.2 kDa *versus* 11.4 kDa) and contains, in contrast to gpD, ten reduced cysteine residues.

With the new library and at lower induction strength, the bacterial survival under selection conditions was reduced dramatically with both antigens, compared to the results from the first set of experiments (Table 1). In order to determine the impact of each of the implemented improvement steps (new antibody library and lower expression level), several additional co-transformations and expression tests were performed (data not shown). A decrease of bacterial growth (one order of magnitude) was observed when the V_H3-$V_\kappa 3$ antibody library was co-transformed instead of

Table 1. Summary of the PCA selection experiments

Applied selection strategy	Library used	Survival rate under selective conditions	No. of antibodies tested for specificity	Specific antibodies identified
GpD fused to wt mDHFRII, competition selection, ten selection rounds, 1 mM IPTG/25 °C	V_H3-$7V_L$	1.0×10^6 cfu	30[a]	D10
GpD fused to wt mDHFRII, single-step selection, 0.1 mM IPTG/25 °C	V_H3-$V_\kappa 3$	1.0×10^3 cfu	50[a]	None
JNK2 fused to wt mDHFRII, single-step selection, 0.2 mM IPTG/25 °C	V_H3-$V_\kappa 3$	1.2×10^2 cfu	80[b]	J21

[a] Specificity verification by ELISA (antibody-mDHFRI fusions, crude cell extracts).
[b] Specificity verification by utilization of PCA (co-transformation of the selected antibodies with the specific antigen and an unrelated antigen).

Table 2. Amino acid sequences of the PCA selected antibodies D10 and J21

VH	Subtype	CDR1	CDR2	CDR3
D10[a]	V_H3	GFTFSSYAMS	AISGSGGSTYYADSVKG	FSYVSGMDY
J21[a]	V_H3	GFTFSSYGMS	NISSDGSNTNYADSVKG	TYIQDF
VL	Subtype	CDR1	CDR2	CDR3
D10[a]	$V_\lambda3$	SGDALGDKYAS	DDSDRPS	QSYDNDFYGT
J21[a]	$V_\kappa3$	RASQSVNSFLA	DASNRAT	QQYNSYPF

[a] Framework sequences of both V_H and V_L domains correspond to the HuCAL® master genes.[27]

the initial antibody library. As this antibody library-dependent effect was observed reproducibly for every antigen-mDHFRII fusion protein utilized, it suggests that we were able to reduce the proportion of misfolded antibodies by using this new antibody library. Furthermore, the expression level of the gpD-mDHFRII fusion protein is significantly higher than the expression level of the JNK2-mDHFRII fusion protein (data not shown), and this may provide another factor why co-transformation of JNK2-mDHFRII with both types of antibody library-mDHFRI fusion yielded one order of magnitude fewer bacterial colonies than the corresponding co-transformations of gpD-mDHFRII. Finally, we found that reduction of the induction strength had the greatest influence on bacterial growth. A tenfold reduction of the concentration of IPTG (from 1 mM to 0.1 mM) yielded a 100-fold impaired bacterial survival rate. In summary, we conclude that bacterial survival under selection conditions depends strongly on the expression level of the mDHFR fusion proteins, and on the quality and stability of the binding molecule library used.

As we aimed to establish PCA for single-step selections, clones from selections with both antigens were characterized directly, and no rounds of consecutive liquid culture selection were performed. From the 1000 bacterial colonies of our second gpD selection, we directly expressed 50 of the selected antibodies in the mDHFRI fusion format and performed again an antigen ELISA with crude extracts of the antibody expression cultures. From this set, no gpD-specific antibody was identified (data not shown). We cannot exclude that it would have been possible to identify gpD-specific antibodies by simply screening more clones.

The specificity of the antibodies resulting from JNK2 selection was tested in the PCA system directly (data not shown). For this purpose, the isolated plasmids of putative JNK2-binding antibodies were co-transformed with the unspecific gpD-mDHFRII antigen construct. We expected no bacterial growth in these control transformations if a particular antibody was specific for JNK2. In contrast, if a particular antibody was an unspecific binder, it probably would induce bacterial growth with every antigen utilized. Out of the 78 JNK2 selected antibodies, 75 induced bacterial growth with the unspecific antigen gpD. Nevertheless, three candidates showed no bacterial growth when co-transformed with gpD-mDHFRII, of which only one (antibody J21) was able to induce bacterial growth after repeated co-transformation with JNK2-mDHFRII. Thus, similar to the initial PCA selection against gpD, one specific antibody resulted from the selection against JNK2 (Table 2).

Expression and protein purification of the PCA-selected scFv antibody fragments

To determine the biophysical and binding properties of the PCA-selected antibodies *in vitro*, the scFv fragments D10 and J21 were re-cloned in a vector that corresponds to the PCA selection plasmid but contains no DHFR fusion. Both scFv antibody fragments were then expressed and purified from the *E. coli* cytoplasm by immobilized metal ion affinity chromatography (Figure 3), either in the presence of 2-mercaptoethanol (to avoid oxidation and obtain the reduced form) or in the absence of reducing agent. For the latter, oxidizing, purification strategy, the formation of disulfide bonds was subsequently induced by air-oxidation, catalyzed by addition of $CuSO_4$. Following this expression and purification protocol, we were able to purify 5–8 mg of the scFv fragment D10 and 8–12 mg of the scFv fragment J21 in functional form from 1 l shake-flask bacterial expression cultures.

In addition, the PCA-selected scFv antibody fragments were re-cloned for expression in the periplasm of *E. coli*. Both scFv antibody fragments were found to be soluble upon periplasmic expression where disulfide formation can occur (Figure 3(b)).

Finally, the purified scFv antibody fragments, which resulted from cytoplasmic expression, were characterized by gel chromatography, equilibrium denaturation experiments, ELISA, and Biacore measurements (see below).

In vivo and *in vitro* specificity verification of the PCA-selected scFv antibody fragments

To determine the *in vivo* specificity of the PCA-selected scFv antibody fragments, the plasmids encoding the gpD-specific antibody D10 and the JNK2-specific antibody J21 were co-transformed with four different antigen-mDHFRII fusion constructs. Besides the respective specific antigens gpD and JNK2, two unrelated antigens (the leucine zipper GCN4[31] and the peptidyl-prolyl *cis*/*trans* isomerase FkpA[32]) were tested. After separate co-transformation of both antibody-encoding plasmids

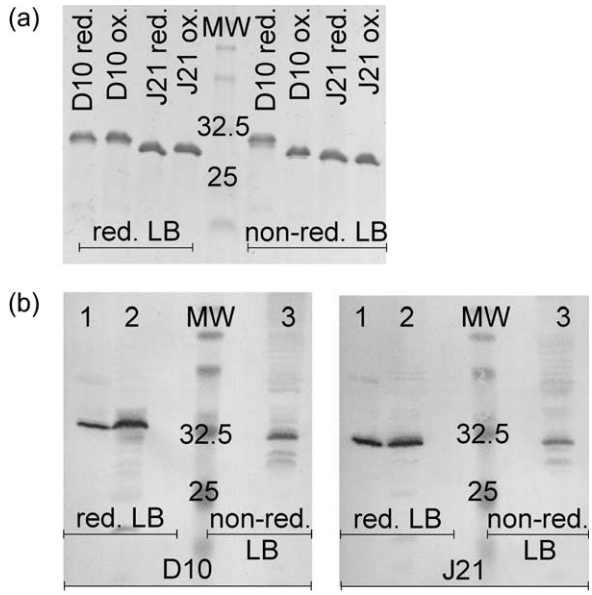

Figure 3. Purification and redox-state analysis of the PCA-selected scFv antibody fragments D10 and J21. Proteins were expressed both in the cytoplasm and in the periplasm of *E. coli* and analyzed by SDS-PAGE (15% polyacrylamide gel). The size marker is indicated in kDa. (a) Redox-state analysis of the cytoplasmically expressed antibody samples, immobilized metal ion affinity chromatography-purified in either the presence of 2-mercaptoethanol to avoid oxidation (reduced samples) or in the absence of any reducing agent. For the latter samples, the disulfide formation was subsequently induced by air-oxidation catalyzed by addition of $CuSO_4$. Samples of soluble elution fractions were separated in the presence (reducing) or in the absence (non-reducing) of 2-mercaptoethanol in the SDS-PAGE loading buffer (LB). Note that under non-reducing conditions the oxidized form of the scFv antibody fragment D10 migrates faster than the corresponding reduced antibody sample, whereas both redox states of the scFv antibody fragment J21 migrate almost identically. (b) Western blot analysis of the periplasmically expressed scFv antibody fragments D10 and J21 (lane 1, insoluble fraction of crude expression lysate; lanes 2 and 3, soluble fraction of crude expression lysate in either reducing or non-reducing loading buffer). Expressed antibody fragments were detected with an anti-FLAG tag M1 antibody. Note that the periplasmically expressed scFv antibody fragments migrate at a higher molecular mass than the cytoplasmically expressed scFv antibody fragments because of their additional FLAG tags (see Materials and Methods).

with all four antigen-encoding plasmids, the transformed *E. coli* cells were incubated in parallel under selective conditions. After three days of incubation we could show that both D10 and J21 exclusively induced bacterial growth with their specific antigen, and no background bacterial growth with any of the unspecific antigens was detectable (Figure 4).

To demonstrate specificity of the PCA-selected scFv antibody fragments *in vitro* as a function of their redox state, we performed ELISA experiments. Binding of D10 and J21 in either reduced or oxidized form to biotinylated gpD, biotinylated JNK2 and bovine serum albumin (BSA) was compared. In this ELISA, both scFv antibody fragments clearly favored binding to their cognate antigen over binding to any unspecific antigen. Interestingly, all applied antibody fractions retained comparable antigen binding regardless of the antibody redox state (Figure 5).

To test whether the antigen binding of the PCA-selected antibody fragments is indeed comparable for both the reduced and oxidized scFv antibody fragments, we additionally determined the dissociation constant of the gpD-specific antibody D10 by Biacore (Figure 6). As expected from our ELISA experiments, the value of the dissociation constant (K_D) obtained for either the oxidized (28.7 μM) or the reduced (30.5 μM) antibody fragment D10 is almost identical. The affinity of the scFv antibody fragment J21 could not be determined accurately by Biacore, but was estimated to be of the order of 50–100 μM.

Analytical gel-filtration chromatography

After purification of the cytoplasmically expressed antibodies, the PCA-selected scFv antibody fragments D10 and J21 in either reduced or oxidized form were subjected to analytical gel-filtration chromatography (Figure 7). Both scFv antibody fragments were determined to be monomeric, independent of their redox state.

Equilibrium denaturation experiments

The thermodynamic stability of the PCA-selected scFv antibody fragments was examined by guanidine hydrochloride (GdnHCl) equilibrium denaturation experiments (Figure 8). Unfolding of the scFv antibody fragments under both non-reducing and reducing conditions was monitored by the shift of the fluorescence emission maximum as a function of the concentration of denaturant after excitation at 280 nm. As the denaturant-induced unfolding was not fully reversible for all the proteins investigated (data not shown), no $\Delta G_{N\text{-}U}$ values are reported; instead, the midpoint of the transition of denaturation are given (Table 3), which is a semi-quantitative measure for the stability of the scFv antibody fragments.

With the knowledge of the denaturation properties of both the isolated V_H and V_L domains and the combinations of these domains in scFv antibody fragments,[26] the resulting antibody fragments can be grouped into different classes.[33] If the intrinsic stability of one domain is significantly higher than the total stability (intrinsic plus interface stabilization) of the other domain, a visible step in the unfolding curve is observed. Both redox states of the scFv antibody fragment D10 showed such an unfolding behavior. The two transitions observed are thus assigned to the unfolding of the separate V_H and V_L domains. If, in contrast, the intrinsic stability of one domain is in the same range as the total stability of the other domain, no step will be

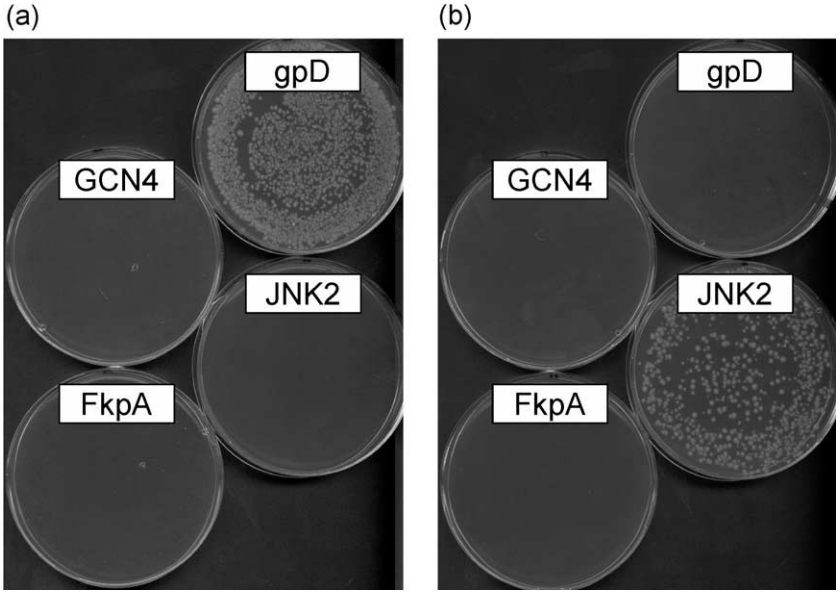

Figure 4. *In vivo* specificity verification of the PCA-selected scFv antibody fragments D10 and J21. (a) Co-transformation of the gpD-specific antibody D10 and (b) the JNK2-specific antibody J21 with their respective cognate (either gpD or JNK2) and two unrelated antigens (leucine zipper GCN4 and peptidyl-prolyl *cis/trans* isomerase FkpA). All co-transformations were plated on selection medium containing 1 mM IPTG or 0.2 mM IPTG and incubated for either 72 h at 25 °C or for 48 h at 30 °C, respectively. Growth is observed exclusively (for all evaluated incubation conditions) as a result of specific interaction between the selected antibodies with their cognate antigens. Here, the selection plates resulting from incubation for 72 h at 25 °C on selection medium containing 0.2 mM IPTG are depicted.

detectable in the equilibrium unfolding curve. Such a single transition was observed for both redox states of the scFv antibody fragment J21. In this case, the midpoint of denaturation is assigned to both the V_H and V_L domains.

As one would expect, both scFv antibody fragments gain stability by disulfide bridge formation. Nevertheless, with midpoints of 1.1 M/2.1 M GdnHCl for D10 and 1.9 M GdnHCl for J21, both scFv antibody fragments possess a respectable thermodynamic stability, even when reduced, compared to other antibody framework subtypes.[26]

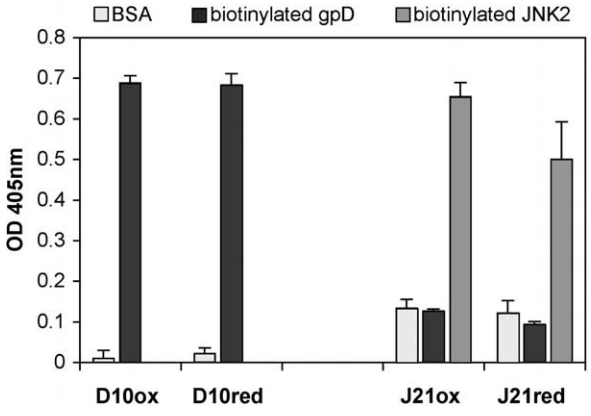

Figure 5. *In vitro* specificity verification of the PCA-selected scFv antibody fragments D10 and J21 by ELISA. Comparative binding of the gpD-specific antibody D10 and the JNK2-specific antibody J21 to gpD, JNK2 and BSA. Antigens were biotinylated and bound to a Neutravidin-coated ELISA plate. ELISAs were performed utilizing 1 μM purified scFv antibody fragment in either the reduced or the oxidized form. Binding of the antibody fragment D10 to gpD is clearly favored over binding to BSA (binding to JNK2 was not evaluated because JNK2 was expressed and purified as gpD fusion protein; see Materials and Methods), whereas the antibody fragment J21 preferentially binds JNK2. In addition, both the reduced and oxidized fractions of each antibody tested retained comparable antigen specificity independent of the antibody redox state.

Discussion

PCA-selected antibodies are effective intrabodies

The PCA-based approach presented here was performed to take advantage of the fast selection possible from a complex library and the absence of a need to express and immobilize the target protein for selection. As a welcome further benefit, this direct *in vivo* selection facilitates the isolation of the antibodies that can fold adequately, have sufficient thermodynamic stability and can function in a reducing intracellular environment. On the basis of these considerations, the scFv antibody fragments D10 and J21 were selected from naive antibody libraries by PCA.

In many cases, intrabodies have been characterized only in terms of *in vivo* functionality, and no biophysical data of the respective antibodies were reported.[34,35] Thus, it is difficult to compare the biophysical properties of the PCA-selected antibody fragments with other intracellularly expressed antibody fragments. However, to summarise, the PCA-selected scFv fragments reported here are resistant to aggregation and can be expressed and purified in soluble form in high yields, especially when compared with the few reported purification yields of other intrabodies.[21,25] The biophysical properties are in line with the expectations from the design of the

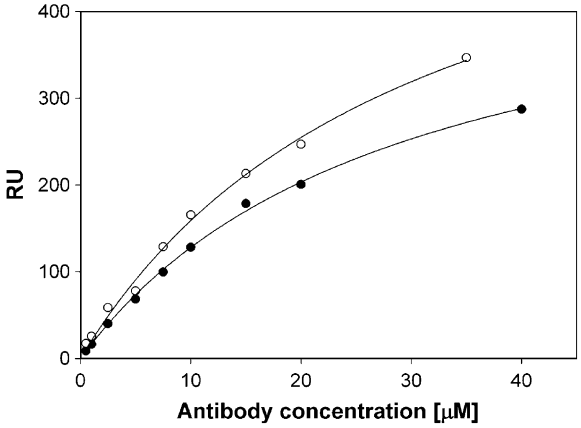

Figure 6. Measurement of the dissociation constant (K_D) of the oxidized (filled circles) and reduced (open circles) scFv antibody fragment D10 for immobilized gpD by Biacore. GpD was covalently bound on a CM5 sensorchip. The applied antibody samples were first injected over a blank and inactivated reference flow-cell. The plateau signal over the reference flow-cell was subtracted from the signal obtained on the gpD-coupled surface to eliminate the contribution of the bulk effect and of possible unspecific binding to the dextran matrix of the sensorchip. K_D was obtained by fitting a hyperbolic curve to the plot of the Biacore plateau signal *versus* the concentration of the injected antibody. The K_D values obtained by this experimental procedure showed an almost identical binding of the oxidized (28.7 μM) and the reduced (30.5 μM) scFv antibody fragment D10 to gpD. Note that the K_D values are strongly dependent on the endpoint of the fitted curve and, therefore, have to be considered as an approximation.

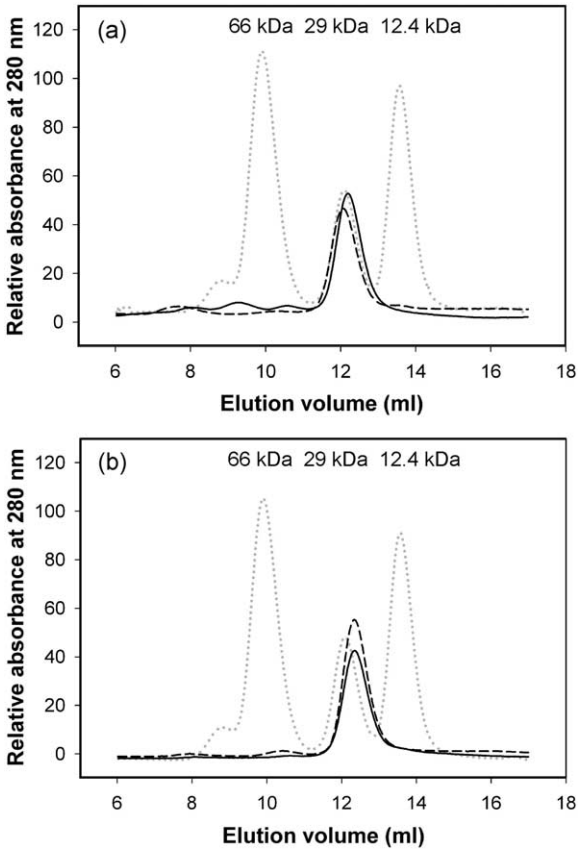

Figure 7. Analytical gel-filtration chromatography of the scFv antibody fragments (a) D10 and (b) J21 in either reduced (broken black line) or oxidized (continuous black line) form. The scFv antibody fragments were injected at a concentration of 15 μM each. All applied proteins are determined to be monomeric, independent of their redox state. The molecular mass standards (BSA, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome *c*, 12.4 kDa) are indicated as dotted grey lines in the graph.

library, as only frameworks with favorable properties were included in the library.[26]

Sequencing of the PCA-selected scFv antibody fragments showed that both D10 and J21 contain no mutation in their framework regions. This implies that the antibodies can tolerate the loss of their intra-chain disulfide bonds, consistent with expectations for stable frameworks.[26,36,37] Indeed, we could show in ELISA and Biacore experiments (performed only for D10) that both scFv antibody fragments retained their functionality in a fully reduced state. This demonstrates that antibodies resulting from PCA are indeed selected for their ability to bind the presented target protein intracellularly. Those rather rare antibodies that are functional despite the lack of disulfide bridge formation must possess an above-average thermodynamic stability as an intrinsic molecular property.[36] Denaturant-induced unfolding experiments finally showed that, indeed, the PCA-selected scFv antibody fragments have a high level of thermodynamic stability. Thus, they compare favorably with other intracellularly functional antibody fragments,[16,23,38] but are also among the more stable of other reported antibody fragments.[26,39]

Given the low affinity observed for the antibody D10, affinity does not seem to determine the outcome of PCA-based selections. This assumption is in agreement with other results that showed that the efficacy of an intrabody is dictated by its intracellular stability rather than by its affinity for the antigen.[38,40] This is most likely due to the fact that at the high intracellular concentrations of antibody and antigen, even for moderately expressed proteins, an antibody–antigen pair of micromolar K_D will already quantitatively form a complex, such that a pair with nanomolar or picomolar K_D gains no further benefit.

Design and intracellular performance of the applied antibody libraries

In order to perform successful intracellular selections of antibody fragments, it has been noted that it would be useful to construct antibody libraries that are more likely to contain a higher percentage of molecules that fold in the reducing milieu.[35] For the design of such antibody libraries, different routes have been followed: either libraries of CDR cassettes were grafted onto frameworks that have been empirically shown to be functional

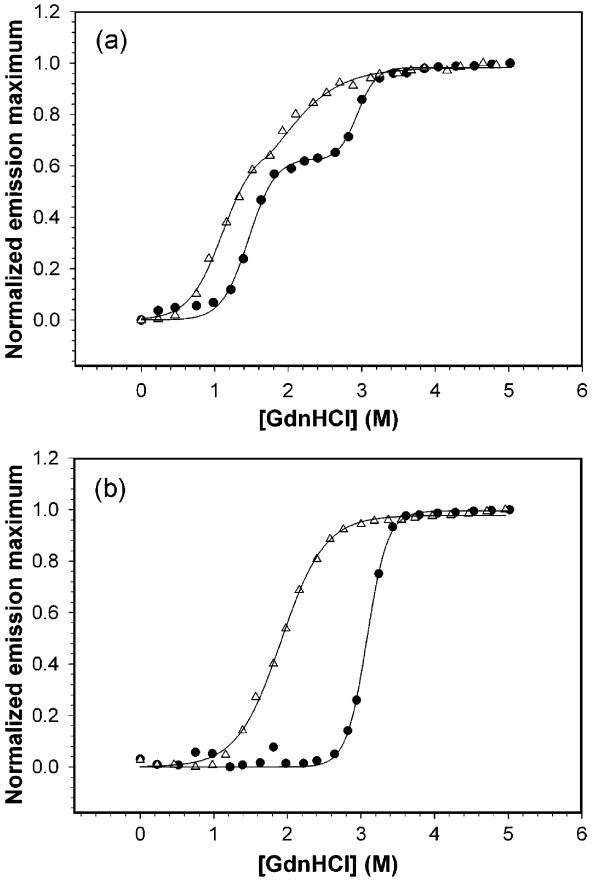

Figure 8. GdnHCl–denaturation curves of the oxidized (filled circles) and reduced (open triangles) scFv antibody fragments (a) D10 and (b) J21. All unfolding transitions were measured following the change in emission maximum as a function of the concentration of denaturant. The fluorescence emission spectra of the samples were recorded from 320 nm to 370 nm, with an excitation wavelength of 280 nm. Normalized maxima refer to a scaling in which the lowest value is set to zero, and the highest value is set to 1. Note that this procedure does not flatten the pre-transition or post-transition baseline. The curves are included only as visual aids.

intracellularly,[21,23] or certain combinations of antibody frameworks have been used that were experimentally found to be more effective in an intracellular environment.[19,24,25,41]

The design of the antibody libraries applied in our PCA selections resulted from a comprehensive biophysical characterization of isolated antibody domains.[26] On the basis of this study, we assembled two antibody libraries in scFv format (V_H3 in combination with all seven HuCAL® V_L domains and V_H3 in combination with only $V_\kappa3$) from the antibody domains possessing the highest thermodynamic stabilities and the highest yield of soluble protein. This strategy was employed to retain intracellular functionality for the majority of the antibody library members. The utilization of antibody domains, adapted to the intracellular selection milieu, might maintain functional diversity, since more library members possess the biophysical requirements. In contrast, the overall performance of such antibody libraries may still be only moderate, as the structural diversity of the different framework classes is a very important factor for maintaining a diversity of antigen-binding sites, which cannot be fully mimicked by single-framework libraries with only CDRs randomized.[42] Furthermore, the structural diversity of the library is one of the most important factors for performance of any selection or directed evolution experiment,[43,44] and the limited diversity among the single frameworks and the subset of superb folding molecules might be one of the reasons for our selection of only a restricted group of intracellular functional antibodies and their moderate affinities.

On the basis of the PCA selections described here, we could show that multiple V_H and V_L frameworks can serve as scaffolds for functional intrabodies. As the gpD-specific antibody D10, composed of V_H3-$V_\lambda3$ domains, was selected from our first antibody library, whereas the JNK2-specific antibody J21 was selected from our second V_H3-$V_\kappa3$ single-framework antibody library, we could demonstrate that both libraries can serve as a source for intracellular functional antibodies. When comparing the *in vivo* performance of both libraries, a much higher bacterial survival rate from co-transformation of our first, multiple V_L domain containing library was found. On the basis of our sequencing results, we suggest that the increased bacterial survival resulted from a predominant enrichment of antibodies comprising the less stable

Table 3. Summary of biophysical characterization of the PCA-selected scFv antibody fragments

Clone	Redox-state	Yield of soluble protein (mg/l culture)[a]	Oligomeric state[b]	Midpoint of unfolding [GdnHCl] (M)	K_D (μM)[c]
D10	Reduced	5–8	Monomer	1.1/2.1[d]	30.5
D10	Oxidized[e]	5–8	Monomer	1.5/2.9[d]	28.7
J21	Reduced	8–12	Monomer	1.9	n.d.
J21	Oxidized[e]	8–12	Monomer	3.1	n.d.

[a] The amount of antibody purified from a 200 ml expression culture was recalculated for a 1 l expresssion culture.
[b] As determined by gel-filtration chromatography.
[c] As determined by Biacore.
[d] Two separate transitions for V_L and V_H.
[e] Cytoplasmic expression, disulfide formation catalyzed by air-oxidation in the presence of $CuSO_4$.

light chain domains $V_\lambda2$ and $V_\lambda3$. These antibodies may not fold properly in the bacterial cytoplasm, therefore leading to bacterial growth through unspecific interactions with the antigen-mDHFRII fusion protein. For this reason, the V_H3-$V_\kappa3$ single-framework antibody library appears a better starting point for forthcoming PCA selections.

Conclusions

Here, we have presented the first successful application of the protein fragment complementation assay (PCA) for the intracellular selection of antibodies from a complex naïve library. Two specific scFv antibody fragments for two independent antigens have been enriched from complex libraries by different selection strategies. The antibody fragments generated show a high soluble expression yield and possess high thermodynamic stability. Furthermore, we could show that antibody functionality does not require disulfide bond formation, but it is also not impeded by it. With these results, a direct *in vivo* selection system for generating binding molecules, and particularly intrabodies, has become available. Nevertheless, its development and application is still at an early stage, with non-specific intracellular binding and lack of reward for high-affinity interactions as remaining challenges to be overcome. The identification of specific binding molecules in only a single PCA selection round would be highly attractive for proteomics projects, and further improvements may bring this technology towards this goal.

Materials and Methods

Construction of mDHFR fusion proteins

Design of the first antibody library

This antibody library has been assembled in the scFv format through combination of four HuCAL (MorphoSys AG, Munich) sublibraries,[27] in order to combine the most stable heavy chain domain (V_H3)[26] with all seven light chain domains. In this library design, only the CDR3 cassettes of both the heavy chain domains and the light chain domains were diversified. The starting point for the construction of the antibody library was the scFv master gene sublibraries of HuCAL® in the orientation V_H-$(Gly_4Ser)_4$-V_L.[27] As recipient vector, the PCA antibody plasmid pHK36, which already contained the HuCAL-derived scFv anti-FkpA, 7B2, was used.[8] First, a fragment of approximately 1 kb was removed from pHK36 by digestion with BsmI. This was done in order to remove several restriction sites, which would be incompatible with the modular design of the HuCAL® antibody library. Then, the modified vector pHK36 as well as the HuCAL® master gene sublibraries were digested with Eco52I and EcoRI. After ligation of the gel-purified scFv antibody fragments in pHK36, the ligation mix was electroporated in *E. coli* XL1-Blue cells (Stratagene). Finally, the constructed antibody library (pHK44) possesses a diversity of 4.5×10^9 transformants, which corresponds to a tenfold over-sampling of the theoretical library size.

Design of the refined antibody library

The second antibody library was assembled in the scFv format through combination of two HuCAL® sublibraries in order to combine the most stable heavy chain domain (V_H3) with the most stable light chain domain ($V_\kappa3$). In this particular library, all six CDR cassettes of both the heavy chain domains and the light chain domains were diversified. The starting point for construction of the second antibody library was the Fab master gene sublibraries of HuCAL Gold (MorphoSys AG, Munich). As recipient vector the BsmI-modified PCA antibody plasmid pHK36 (see construction of the first antibody library), which already contained the HuCAL-derived scFv anti-FkpA, 7B2 was used.[8] First, the anti-FkpA antibody, 7B2, present in the vector, was removed from pHK36 and replaced by a HuCAL®-derived dummy V_H3-$(Gly_4Ser)_4$-$V_\kappa3$ scFv antibody fragment. Additionally, the restriction site BspEI, which would have been incompatible with the intended cloning strategy, was removed by site-directed mutagenesis from the linker between the scFv and the mDHFRI fragment. All listed modifications of pHK36 finally yielded the vector pHK46, which is fully compatible with the flanking restriction sites of the HuCAL® Gold V_H3 and the $V_\kappa3$ sublibraries. First, both pHK46 and the HuCAL® Gold $V_\kappa3$ sublibrary were digested with PstI and MscI. After ligation of the gel-purified $V_\kappa3$ sublibrary in pHK46, the ligation mix was electroporated in *E. coli* XL1-Blue cells (Stratagene). This particular cloning step finally yielded the vector pHK47, which still contained the V_H3 dummy domain plus the $V_\kappa3$ sublibrary with a diversity of 5.4×10^7 transformants. After construction of the $V_\kappa3$ sublibrary the vector pHK47 and the HuCAL® Gold V_H3 sublibrary were digested with BspEI and Bpu1102I. Similar to the construction of the $V_\kappa3$ sublibrary, the gel-purified V_H3 sublibrary was first ligated to pHK47, and afterwards electroporated in *E. coli* XL1-Blue cells (Stratagene). Finally, the constructed single-framework (V_H3-$V_\kappa3$) antibody library (pHK48) possesses a diversity of 3.8×10^9 transformants, which represents no over-sampling of the theoretical library size.

Design of the antigen constructs

First, the β-lactamase resistance cassette of the antigen-encoding plasmid pHK8[8] was replaced by an optimized chloramphenicol resistance cassette[45] (engineered to eliminate several restriction sites), yielding the plasmid pHK40. Thus, both the antibody and antigen-encoding plasmids contain different resistance genes in order to allow an efficient separation of the plasmids after a selection has been performed.

In order to exchange the antigen of the antigen vector pHK40, gpD was PCR-amplified from the plasmid pAT101,[28] using the following oligonucleotides: forward 5′-GGA TCC GCA TGC TTG CGA GCA AAG AAA CCT TTA CC-3′ and backward 5′-CAT CTT TAT AGT CGA ATT CAA CGA TGC TGA TTG CCG TTC C-3′. After digestion of both the resulting PCR fragment and the vector pHK40 with SphI and EcoRI the antigen gpD-encoding fragment was gel-purified and ligated in pHK40, finally yielding the plasmid pHK43. JNK2α2 was PCR amplified from the plasmid pAT222_JNK2[46] using the following oligonucleotides: forward 5′-GCT CAG GCA TGC TTT CCG ACT CTA AAT GTG ACA G-3′ and backward 5′-GCT GCA GAA TTC TCG ACA GCC

TTC AAG GGG-3′. After digestion of both the resulting PCR fragment and the vector pHK43 with SphI and EcoRI the antigen JNK2-encoding fragment was gel-purified and ligated in pHK43, finally yielding the plasmid pHK92.

Protein fragment complementation assay

Electro-competent *E. coli* BL21/pRep4 cells (transformation efficiency $=1\times10^9$/μg of DNA for selection experiments and at least $\geq3\times10^8$/μg of DNA for specificity verification) were co-transformed with 100 ng of each the antibody-mDHFRI and the antigen-mDHFRII fusion protein-encoding plasmids. After incubation for 1 h at 37 °C (no selection pressure), the cells were washed with M9 minimal medium and either transferred to M9 medium containing 50 μg/ml of kanamycin (kan^R, repressor plasmid pRep4), 100 μg/ml of ampicillin (amp^R, antibody library-encoding plasmid), 10 μg/ml of chloramphenicol (cam^R, antigen-encoding plasmid), 2 μg/ml of trimethoprim (to inhibit bacterial DHFR and select for functional mDHFR), and various concentrations (100 μM to 1 mM) of IPTG (induction of both mDHFR fusion proteins). By adding 5% (w/v) of Casamino acids (Difco) to the standard composition of M9 minimal medium, the incubation time on solid selection medium was reduced to 72 h at 25 °C or 48 h at 30 °C.

For performance of successive selection rounds in liquid culture, 75 ml of selection medium (composition as above) was inoculated from the respective preculture to a final $A_{600}=0.001$. Expression of the mDHFR fusion proteins was induced directly by addition of IPTG to a final concentration of 1 mM. Each of the ten selection rounds was performed for 12 h at 25 °C.

For *in vivo* specificity determination of both scFv antibody fragments D10 and J21, selection plates containing 1 mM or 0.2 mM IPTG were utilized. These particular selection plates were incubated for 72 h at 25 °C and 48 h at 30 °C, respectively.

Isolation of antibody-encoding plasmids

After performance of a PCA selection experiment, the antibody-encoding plasmids had to be isolated to verify the antigen-specificity of each individual antibody. Since overnight cultures inoculated from the selection plates were expected to contain three plasmids, the antibody-encoding plasmid had to be separated from the repressor plasmid pRep4 and the antigen-encoding plasmid. After preparation of DNA from the selection plate, a small sample of the miniprep elution fraction was heavily over-digested with either RsrII (gpD selection) or Bsp120I (JNK2 selection). These restriction sites were present exclusively in the repressor plasmid pRep4 and the antigen-encoding plasmid. Therefore, the antibody-encoding plasmids remain undigested. In contrast, if a selected antibody gene would contain also one (or multiple) restriction site (v) of the chosen enzymes, a particular antibody clone would be lost. This might have been the reason for the two observed antibody gene deletions in the gpD-selection experiment. After re-transformation of the digested DNA sample into *E.coli* XL-10 cells (Stratagene), the transformation mix was plated on LB-agar plates (2% (w/v) glucose; 100 μg/ml of ampicillin), and one resulting bacterial colony of each individual antibody construct was used for repeated DNA isolation. Finally, the purity of the antibody-encoding plasmid was verified by EcoRI/SphI restriction digestion of a proportion of the latter DNA isolation.

Expression of selected antibody-mDHFRI fusion proteins

After isolation of the antibody-encoding plasmid, *E. coli* BL21/pRep4 (Qiagen) cells were transformed with the vectors. 50 ml of 2YT medium containing 50 μg/ml of kanamycin and 100 μg/ml of ampicillin was inoculated with an overnight preculture to a final $A_{600}=0.1$. Expression was induced at an A_{600} of approximately 0.8 by addition of IPTG to a final concentration of 1 mM. Expression was performed for 4 h at 25 °C.

Antigen preparation for *in vitro* specificity verification

For expression of the antigen constructs the vectors pAT222 (GenBank accession no. AY327137) and pAT222_JNK2 were used. Expression from these vectors yields a fusion protein comprising an N-terminal avi-tag for *in vivo* biotinylation, bacteriophage lambda protein gpD, followed by JNK2 (in the case of the vector pAT222_JNK2), and a C-terminal His_6 tag for purification (avi-gpD-(JNK2)-His_6). Both gpD and JNK2 were expressed, biotinylated and purified as described.[46] The purity of the samples was checked by SDS-PAGE analysis and the concentration was determined by measuring the absorbance at 280 nm.

In vitro specificity verification of PCA-selected antibodies by ELISA

Biotinylated gpD or biotinylated JNK2 (100 μl/well; 10 μg/ml) was bound to a neutravidin-coated (100 μl/well; 5 μg/ml) ELISA plate (MaxiSorp). After being washed with Tris-buffered saline (TBS; 20 mM Tris–HCl (pH 7.5), 150 mM NaCl) containing 0.05% (v/v) Tween, the plates were blocked for 1 h with 300 μl/well of TBS, 0.5% (w/v) BSA. ELISAs were performed either with crude extract supernatant (150 μl/well) of the antibody-mDHFRI fusion proteins (initial PCA selection, screen for gpD binders) or with 1 μM purified scFv antibody fragment (redox state-dependent binding of D10 and J21). Binding of the PCA-selected scFv antibody fragments was analyzed by detection of their N-terminal RGS-His_6 tag of either the antibody-mDHFRI fusion proteins or the purified antibody fragments as described.[46]

Construction of the cytoplasmic expression vectors

The PCA-selected scFv antibody fragments D10 and J21 were cloned *via* SphI and HindIII into the standard expression vector pQE32 (Qiagen). The vector pQE32 basically corresponds to the PCA selection plasmid but does not contain any mDHFR fusion protein. It contains an N-terminal RGS-His_6 tag, which was used for subsequent purification of the scFv antibody fragments. Both antibodies were amplified by PCR using the following oligonucleotides: forward (used for the amplification of both antibodies) 5′-GGA TCC GCA TGC TTG AAG TGC AAT TGG TGG AAA G-3′ and backward (for the antibody D10) 5′-GAG GAT CCA AGC TTC TAT TAC TGG CCA AGA ACG GTT AAC TTC-3′ (or the antibody J21) 5′-GAG GAT CCA AGC TTC TAT TAC GTA CGT TTA ATT TCA ACT TTC G-3′.

Cytoplasmic expression of the PCA-selected scFv antibody fragments

All expression experiments were carried out using *E. coli* BL21/pRep4 (Qiagen) cells. Medium (1 l of 2YT medium in a 5 l flask without baffles, 150 rpm) containing 50 μg/ml of kanamycin and 100 μg/ml of ampicillin was inoculated with an overnight preculture to a final A_{600} = 0.1. Cytoplasmic expression of the scFv antibody fragments D10 and J21 was induced at an A_{600} of approximately 0.8 by addition of IPTG to a final concentration of 0.5 mM. Expression was allowed to continue for 4 h at 25 °C. Expression cultures were harvested by centrifugation in portions of 200 ml each. Resulting cell pellets were stored at −80 °C.

Construction of the periplasmic expression vectors

The PCA-selected scFv antibody fragments D10 and J21 were cloned *via* BspEI and EcoRI into the expression vector pMx7 (Morphosys). In pMx7, the scFv antibody fragments are expressed under control of the inducible *lac* promoter/operator and secreted to the periplasm. The final expression cassette consists of a *phoA* signal sequence, the short FLAG tag (DYKD), the scFv antibody fragment in the orientation V_H-$(Gly_4Ser)_4$-V_L, the long FLAG tag (DYKDDDD) and a His_6 tag.

Periplasmic expression of the PCA-selected scFv antibody fragments

All expression experiments were carried out using *E. coli* SB536 cells.[47] Medium (1 l of 2YT medium in a 5 l flask without baffles, 150 rpm) containing 25 μg/ml of chloramphenicol and 50 mM K_2HPO_4 was inoculated with an overnight pre-culture to a final A_{600} = 0.1. Periplasmic expression of the scFv antibody fragments D10 and J21 was induced at an A_{600} of approximately 0.8 by addition of IPTG to a final concentration of 0.5 mM. Expression was allowed to continue for 4 h at 25 °C. Expression cultures were harvested by centrifugation in portions of 200 ml each. Resulting cell pellets were stored at −80 °C until Western blot analysis was performed.

Immobilized metal ion affinity chromatography purification of the cytoplasmically expressed scFv antibody fragments

After cell lysis by French press, the crude extracts of the PCA-selected scFv antibody fragments D10 and J21 were centrifuged at 20500 rpm in an SS34 rotor for 60 min at 4 °C. After passing the supernatant through a 0.22 μm pore size filter, 20 ml of French press lysate supernatant was loaded onto an equilibrated Protino® Ni-2000 column (Machery-Nagel) to purify the scFv antibody fragments *via* their N-terminal RGS-His_6 tag. To remove unspecific binding proteins, the columns were washed with ten column volumes of lysis buffer (50 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl) and five column volumes of high-salt buffer (50 mM Na_2HPO_4 (pH 8.0), 900 mM NaCl). The scFv antibody fragments were eluted by adding two column volumes each of 20 mM imidazole, 50 mM imidazole and 100 mM imidazole. Both scFv antibody fragments were purified following the same protocol. In the case of the reduced antibody samples, 20 mM 2-mercaptoethanol was included in all buffers to avoid oxidation of the scFv antibody fragments.[16] The formation of disulfide bonds in the purification of the oxidized scFv antibody fragments was catalyzed by air-oxidation in the presence of 10 μM $CuSO_4$.[37] The purity of the samples was checked by SDS-PAGE analysis and the concentration was determined by measuring absorbance at 280 nm. The purification was always done for only 200 ml of the 1 l expression culture. The amount of scFv antibody fragment, which was purified from 200 ml of the expression culture, was finally recalculated to refer the total protein yield to that from a 1 l expression culture.

Western blot analysis

For immunoblots, the crude extracts of the periplasmically expressed scFv antibody fragments were analyzed on SDS/15% (w/v) polyacrylamide gels and electro-transferred onto nitrocellulose membranes (Millipore). The PCA-selected antibodies were detected with an anti-FLAG tag M1 antibody (Sigma). An alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) was used as secondary antibody. The immunoblots were developed using the BCIP/NBT color development solution (Biorad) according to the instructions of the supplier.

Gel-filtration chromatography

Samples of purified scFv antibody fragments were analyzed on a Superdex-75 gel-filtration column (ÄKTA, Amersham-Pharmacia) at a concentration of 15 μM each, with 300 mM NaCl, 50 mM Na_2HPO_4 (pH 7.5) and either 10 μM $CuSO_4$ in the case of the oxidized antibodies or 2 mM DTT for the reduced antibodies as running buffer. Proteins were injected in a volume of 100 μl, and the column was run with a flow-rate of 500 μl min^{-1}. Cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), and BSA (66 kDa) were used as molecular mass standards. Elution was followed by detection of the absorbance at 280 nm.

Equilibrium denaturation experiments

Fluorescence spectra were recorded at 20 °C with a PTI Alpha Scan spectrofluorimeter (Photon Technologies, Inc.). Protein/GdnHCl mixtures (1.5 ml) containing a final protein concentration of 0.5 μM and denaturant concentrations ranging from 0 M to 5 M GdnHCl were prepared from freshly purified protein and a GdnHCl stock solution (6 M GdnHCl in 50 mM Na_2HPO_4 (pH 7.5), 150 mM NaCl; in the case of the reduced antibodies, 2 mM DTT was added). Each final concentration of GdnHCl was determined from its refractive index. After incubation for two days at 4 °C, the fluorescence emission spectra of the samples were recorded from 320 nm to 370 nm, with an excitation wavelength of 280 nm. The fluorescence emission maximum, which was determined by fitting the fluorescence emission spectrum to a Gaussian function was plotted *versus* the concentration of GdnHCl. To compare the denaturation curves of the oxidized or reduced scFv fragments in one plot, the emission spectra were normalized by setting the highest values to 1 and the lowest to zero.

Surface plasmon resonance

Surface plasmon resonance (SPR) was measured using a BIAcore 3000 instrument (BIAcore). The running buffer was 10 mM Hepes (pH 7.5), 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20 (in the case of the reduced scFv antibody fragment, 2 mM DTT was added). Biotinylated

gpD was immobilized on a CM5 chip (BIAcore) to 320 resonance units (RU) by amine-coupling as described in the BIAapplications Handbook. The interactions were measured at a flow rate of 5 μl min^{-1}. Pulses (10 μl) of either oxidized or reduced scFv antibody fragment D10 were injected in various concentrations (0.5 μM to 40 μM), followed by a dissociation time of 900 s for the oxidized scFv antibody fragment and 1200 s for the reduced scFv antibody fragment to allow regeneration. The signal at the plateau was calculated by subtracting the signal obtained on a deactivated control surface from the signal obtained on the gpD-coupled surface. Each data point was the average of two measurements. The dissociation constant was obtained by plotting (SigmaPlot 2001, SPSS Inc.) the signal (in RU) against the concentration of the injected antibody (AB), and fitting to a hyperbolic curve:

$$RU = RU_{max}[AB]/(KD + [AB])$$

where RU_{max} is the maximal value at saturation.

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