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Efficient Selection of DARPins with Sub-nanomolar Affinities using SRP Phage Display

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There is an ever-increasing demand to select specific, high-affinity binding molecules against targets of biomedical interest. The success of such selections depends strongly on the design and functional diversity of the library of binding molecules employed, and on the performance of the selection strategy. We recently developed SRP phage display that employs the cotranslational signal recognition particle (SRP) pathway for the translocation of proteins to the periplasm. This system allows efficient filamentous phage display of highly stable and fast-folding proteins, such as designed ankyrin repeat proteins (DARPins) that are virtually refractory to conventional phage display employing the post-translational Sec pathway. DARPins comprise a novel class of binding molecules suitable to complement or even replace antibodies in many biotechnological or biomedical applications. So far, all DARPins have been selected by ribosome display. Here, we harnessed SRP phage display to generate a phage DARPin library containing more than 10^{10} individual members. We were able to select well behaved and highly specific DARPins against a broad range of target proteins having affinities as low as 100 pM directly from this library, without affinity maturation. We describe efficient selection on the Fc domain of human IgG, TNFα, ErbB1 (EGFR), ErbB2 (HER2) and ErbB4 (HER4) as examples. Thus, SRP phage display makes filamentous phage display accessible for DARPins, allowing, for example, selection under harsh conditions or on whole cells. We envision that the use of SRP phage display will be beneficial for other libraries of stable and fast-folding proteins.

Keywords: phage display; library generation; SRP phage display; DARPin; novel scaffold

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Introduction

The demand for high-quality binding molecules for biotechnological and biomedical applications is ever increasing. Antibodies are currently by far the most frequently used binding molecules, but proteins based on non-immunoglobin scaffolds have become an appealing alternative.[1] From both antibody and non-antibody scaffolds, binding molecules with high affinity and high specificity against almost any chosen target protein can be generated *in vitro* using appropriately designed combinatorial libraries in combination with selection technologies such as phage display or ribosome display.[1–4] The success of such selections is based mainly on the design and size of the combinatorial library and the selection technology employed. Together, these factors will determine the functional size of the library that is accessible for the selection of binding molecules. Further important factors are the selection strategy chosen and the biophysical properties of the target protein; even with the best library and an optimized selection protocol it might be very difficult or even impossible to obtain useful binding molecules against a "sticky" and unstable target protein.

The underlying principle of all selection technologies is the physical linkage of the phenotype (i.e. the displayed protein) and the genotype (i.e. the DNA

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Abbreviations used: DARPin, designed ankyrin repeat protein; EGFR, epidermal growth factor receptor; ErbB, erythroblastic leukemia viral oncogene homolog; HER2, human epidermal growth factor receptor homolog 2; POI, protein of interest; SRP, signal recognition particle; TNFα, tumor necrosis factor α.

encoding the displayed protein). Different selection technologies use different strategies to achieve this linkage. The success of any selection experiment depends on the compatibility of the strategy used with the proteins to be displayed. Ribosome display, for example, is a complete *in vitro* display technology that relies on the formation of a non-covalent ternary complex of mRNA, ribosome and the displayed nascent polypeptide.[5] The formation of this ternary complex by *in vitro* translation allows the sampling of very large libraries, and the ease of introducing mutations by error-prone PCR allows efficient affinity maturation. However, the linkage of the genotype and the phenotype is reliably maintained only under non-denaturing buffer conditions, in the absence of RNases and at low temperature. In contrast, in filamentous phage display,[4,6] this linkage is very robust, allowing a broader range of selection conditions. Filamentous phage display achieves this robustness by physically linking the displayed protein to the surface of a very stable phage particle,[7] which encapsulates the corresponding genotype. Nevertheless, the limiting factor in this selection technology is the involvement of an *in vivo* step to produce the protein displaying phage particles. The bacterial transformation needed therefore limits the library size that can reasonably be achieved to usually much less than 10^{11} members. Furthermore, the *in vivo* assembly process of the phage particle is not necessarily compatible with any given protein to be displayed, since its successful translocation to the periplasm and its folding are also prerequisites. In an ideal selection experiment, high-affinity binders should be obtained directly from the library, without further time-consuming affinity maturations.

The success of a phage selection experiment depends strongly, as a first parameter, on the size of the transformed library. In general, the larger the library, the higher the probability of isolating diverse binders against a given target protein, and the higher the affinities of the selected binders.[8] For example, libraries with more than 5×10^9 members were employed to isolate antibody fragments with sub-nanomolar affinities.[9–11] Nevertheless, even more important than the size of the transformed library (number of independent clones), is the functional library size; i.e. the number of different and correct (i.e., full length and folded) molecules available for selection, as often only a fraction of library members can be functionally displayed. Three main factors influence this functional diversity: the above-mentioned compatibility of the selection system with the protein to be displayed; the actual library design; and the size and quality of the assembled library.

In the case of libraries of designed binding molecules, the use of a very stable scaffold as the starting point to introduce diversity helps to ensure that almost all library members are stable and well-expressed proteins, as diversification of a scaffold will almost inevitably result in reduced stability in some library members. Another important aspect is the design of the diversity, meaning the careful design of randomized parts into the scaffold; the challenge is to introduce a level of diversity high enough to allow successful selection of binders against any target protein, yet low enough to not destabilize library members too much.

In the case of designed ankyrin repeat proteins (DARPins),[12,13] which comprise a novel class of binding proteins, we used consensus design to generate a very stable scaffold amenable to the introduction of the needed diversity.[14] This consensus design is based on the sequence and structure analysis of natural ankyrin repeat proteins and allows the delineation of framework residues, which are important for stability of the scaffold, and the definition of potential target interaction residues (inspired by the study of natural ankyrin complexes) suitable to introduce the needed level of diversity. Indeed, most members of such DARPin libraries have very favorable biophysical properties.[13] They are very well expressed, monomeric in solution, highly soluble, thermodynamically stable and show fast cooperative folding behavior.[15]

From such DARPin libraries, specific binders with high affinities have been selected for a broad range of target proteins by using ribosome display.[16–20] Nevertheless, and much to our initial surprise, the display of DARPins on filamentous phage was highly inefficient using conventional systems, despite the good biophysical properties of the proteins. We identified this problem as one of inefficient translocation of the DARPins into the periplasm due to their premature folding in the cytoplasm when using the post-translational Sec pathway, and one that can be solved by using the signal recognition particle (SRP) translocation pathway for phage display.[21]

In SRP phage display,[21] the proteins to be displayed are directed to the *Escherichia coli* cotranslational SRP translocation pathway by using an appropriate signal sequence, whereas conventional phage display uses the post-translational Sec translocation pathway. This simple change in the use of the translocation route by changing the signal sequence seems to prevent premature folding of stable and fast-folding proteins in the cytoplasm and therefore allows their efficient translocation and subsequent display on filamentous phage particles. These results indicated that the use of SRP phage display may help to obtain phage DARPin libraries with high functional diversity, leading to efficient selections.

Here, we describe the generation of a large phage DARPin library by using SRP phage display, selections on a panel of target proteins using this library and analysis of the selected DARPins. In addition, we discuss the potential of SRP phage display to generate novel binding molecules with very desirable properties for biotechnological or biomedical applications.

Results

Construction of the phage DARPin library

The phage DARPin library described here is based on the phagemid vector pPDV1 that allows the dis-

play of a protein of interest (POI) fused to the minor phage coat protein 3 (p3) on filamentous phage (Supplementary Data Fig. 1). In contrast to most other phage display vectors, it fuses the POI to a signal sequence using the cotranslational SRP pathway for translocation of the POI-p3 fusion across the cytoplasmic membrane of *E. coli*. The use of the SRP pathway ensures the efficient display of stable and fast-folding DARPins on phage particles.[21]

We used the N3C DARPin library[13] (N3C denotes an N-terminal capping repeat, three randomized internal repeats, and a C-terminal capping repeat) and brought it to the phage display format by PCR amplification and subcloning it into pPDV1. *E. coli* XL1-Blue cells were transformed with the library yielding 2.6×10^{10} independent colonies (transformed library size; see Material and Methods for details). The transformed *E. coli* XL1-Blue cells were infected with helper phage VCS M13, and subsequently IPTG was added to the culture to induce the expression and thus the display of the DARPins on the phage particles. For all initial rounds of selection, as described below, the input library phage particles were used directly from a frozen phage library stock. There was no need to re-amplify the library to obtain functional display, as the DARPins displayed on phage particles seem to tolerate at least one freeze–thaw cycle very well.

High-level functional diversity of the phage DARPin library

The functional diversity of the transformed 2.6×10^{10} independent clones of the DARPin library was assessed by sequencing, determining the display level and subsequent cytoplasmic expression analysis. DNA sequencing of 55 randomly picked clones showed that 26 (47%) encode functional DARPin sequences, composed of 18 (33%) N3C, 7 (13%) N2C and 1 (2%) N1C DARPin library members, all with different amino acids at randomized positions. The presence of library members having less than three internal repeats (N1C and N2C) can be attributed to *in vitro* recombination during library construction, requiring the PCR amplification of the DARPins, and result from their repetitive nature. Importantly, DARPins of different length can all be functional. The remaining 29 (53%) clones had frameshifts, deletions or insertions in the DARPin sequence or contained no insert at all (non-functional part of the library). Phage particles produced from the same 55 clones of the library as sequenced above were analyzed for DARPin display by Western blot. All 26 (47%) clones correct at the sequence level showed bands at the size expected for a DARPin-p3 fusion protein and signal intensities similar to the signal obtained for a well-folding antibody scFv fragment using a standard phagemid for monovalent display. From this comparison, it can be assumed that, with the current system, single DARPins are displayed on about 1–10% of phage particles.[22] This is a good balance between an efficient yet largely monovalent display.

To analyze cytoplasmic expression of the DARPins, a pool of DARPin inserts from the library was subcloned into pDST67 (see Materials and Methods), a vector for high-level, soluble cytoplasmic expression derived from pQE30 (QIAgen), followed by small-scale expression in 96-deep well plates and SDS-PAGE analysis of 57 randomly picked clones that were not sequenced. Thirty-two (56%) of the clones showed expression levels of soluble DARPins above 0.1 mg/ml *E. coli* culture. Together, these results showed that all clones that are correct at the amino acid level are well displayed on the phage particles and can be expected to be well expressed in the cytoplasm. Thus, we can estimate that the phage DARPin library contains about 1.2×10^{10} correct clones, which encode well-behaved displayable molecules (functional library size, 47% of the transformed library size), taking into account that the library used as template contained at least 10^{10} individual members.[16]

As with any randomized protein library, the non-functional part of the library may sometimes lead to the enrichment of background binding clones encoding defective molecules that have parts of the scaffold replaced by unstructured and sticky polypeptides. In all cases, this background enrichment could be prevented by applying the optimized selection protocol described in detail in the Supplementary Data.

Target protein preparation, immobilization and optimized selection protocol

For the validation of the phage DARPin library, we performed selections against a broad range of purified target proteins (Table 1). To avoid partial protein denaturation of the target due to direct immobilization on solid plastic (i.e. polystyrene) surfaces, samples of all target proteins were chemically or enzymatically (using an Avi-tag [23]) biotinylated. This allows selections to be performed either on biotinylated target protein bound to neutravidin or streptavidin, which are immobilized directly on a solid plastic surface (referred to as an "immobilized target protein") or on biotinylated target protein in solution, which was subsequently captured with streptavidin-coated beads (referred to as a "target protein in solution"). From the analysis of a number of selection experiments, we found that the most efficient enrichment of specific binders from our phage DARPin library is obtained with an optimized selection protocol that is composed of a first round of selection on immobilized target protein, followed by one or two further rounds of selection on target protein in solution. (The development of this optimized selection protocol is described in the Supplementary Data.) Unless stated otherwise, selections were done at room temperature. Enzyme-linked immunosorbent assay (ELISA) experiments were performed on biotinylated target protein bound to neutravidin, which had been immobilized directly on a solid plastic surface, and by using non-biotinylated target protein for competition experiments. The different selection approaches need-

Table 1. Description of target proteins used for the selections[a]

Target protein	Abbr.	Selected binder[b]	Description[c]	Accession number[d]
Fc of huIgG1	huIgG1_Fc	I_xx	P100 – K330 of human IgG1 (Fc domain)	SP:P01857
TNFα	TNFα	T_xx	A76– L233 of human tumor necrosis factor precursor (TNFα)	SP:P01375
ErbB1 (1–501)[e]	ErbB1	E_xx	L25 – S525 of human receptor tyrosine-protein kinase ErbB1 fused to huIgG1_Fc	SP:P00533
ErbB2 (1–631)[e]	ErbB2–631	H_xx	S22 – T652 of human receptor tyrosine-protein kinase ErbB2	SP:P04626
ErbB2 (1–509)[e]	ErbB2–509	9_xx	S22 – N530 of human receptor tyrosine–protein kinase ErbB2	SP:P04626
ErbB4 (1–500)[e]	ErbB4	B4_xx	Q26 – R525 of human receptor tyrosine–protein kinase ErbB4 fused to huIgG1_Fc	SP:Q15303

[a] Abbreviations used: Fc, fragment crystallizable; IgG1, immunoglobulin G1.
[b] Nomenclature of selected binders, xx corresponds to the number of a specific binder.
[c] The first and last amino acid used as denoted in the respective Swiss-Prot reference are indicated in single-letter amino acid code.
[d] Accession number: SP, Swiss-Prot.
[e] ErbB receptors and in parentheses the amino acids of the mature extracellular domain as described.[47]

ed for the different target proteins and characterizations of the binders obtained are described below. This partial listing illustrates the versatility of the library, and the great influence of the target protein on the peculiarities of the selection. While high-affinity and specific binders could be obtained in every case, we chose these particular examples to illustrate our characterization, as well as some challenges and solutions to problems.

Efficient selection of DARPins specific for huIgG1_Fc

Binders against the Fc domain of antibodies are of interest as an in vitro detection reagent, and for biomedical applications (e.g., to extend the *in vivo* half-life of molecules fused to such a binder by attaching to antibodies present in the blood stream). To enrich binders against the Fc domain of human IgG1 (huIgG1_Fc, Table 1) we performed three rounds of selection using our optimized selection protocol as described above. From each selection round we analyzed the recovered and amplified polyclonal pool of phage particles for target binding by phage ELISA. Enrichment of phage particles displaying target specific DARPins was already observed after the second selection round with a strong increase after the third round, and no background enrichment of phage particles binding to the corresponding non-target-coated matrix was observed (Fig. 1a).

To screen individual DARPins for binding to their target protein, and at the same time eliminate any conceivable dependence on the p3-fusion format, we did not carry out single-clone analysis with phages but subcloned directly the selected pool of binders from round 3 into the cytoplasmic expression vector pDST67. From 94 individual DARPins analyzed for full-length huIgG1 binding by a crude extract ELISA (see Materials and Methods), 82 gave a specific binding signal (signal/background > 10) (Fig. 1b; Table 2). Crude cell extracts of ELISA-positive clones were further analyzed by SDS-PAGE (Fig. 1c), confirming that, in general, the excellent expression properties of DARPins are maintained throughout. It should be noted that some DARPins having more than two internal repeats run at lower apparent molecular mass or show additional bands with faster running behavior. The same behavior was observed for the respective purified proteins, even though they were subsequently shown by DNA sequencing and mass spectrometry to be precise in sequence and not degraded. As shown previously,[13,21] this is due to an incomplete denaturation of these stable molecules by SDS in the loading buffer, even after boiling. N2C DARPins do not have this extreme SDS-resistance and do migrate where expected, consistent with the observed increase in stability with the number of repeats.[15] On the basis of the ELISA signals, 21 clones were submitted for sequencing, resulting in eight different sequences of DARPins confirmed by replicate ELISA experiments to bind the target protein (data not shown). The six most promising candidates (Table 2), named I_02, I_07, I_13 (N2C library members), and I_01, I_11, I_19 (N3C library members) were analyzed further. Expression and immobilized metal ion chromatography (IMAC) purification was performed in 96-well format in parallel with binders selected against other target proteins (see Materials and Methods), yielding on average more than 1 mg of > 95% pure protein for each clone from 8 × 1.3 ml *E. coli* culture. These purified DARPins were used for all further experiments.

To investigate the specificity of the selected DARPins, ELISA experiments with the four high-affinity clones I_01, I_02, I_07 and I_19 (Fig. 1c) and the two lower-affinity clones I_11 and I_13 (Fig. 1d) were performed. All of the binders do show specific binding to full-length human IgG1 (huIgG1) but do not bind to neutravidin, mouse IgG1 (muIgG1) or mouse IgG2b (muIgG2b). In the competition ELISA setup, the binding to immobilized huIgG1 could be well inhibited by preincubation of the DARPins with free huIgG1 (Fig. 1c and d). From the concentrations of free huIgG1 needed for an inhibition of > 50%, the affinities of these binders can be estimated to be in the nanomolar range, as could be confirmed by surface plasmon resonance (SPR, see below).

The binders were further characterized by size-exclusion chromatography (SEC) and compared to

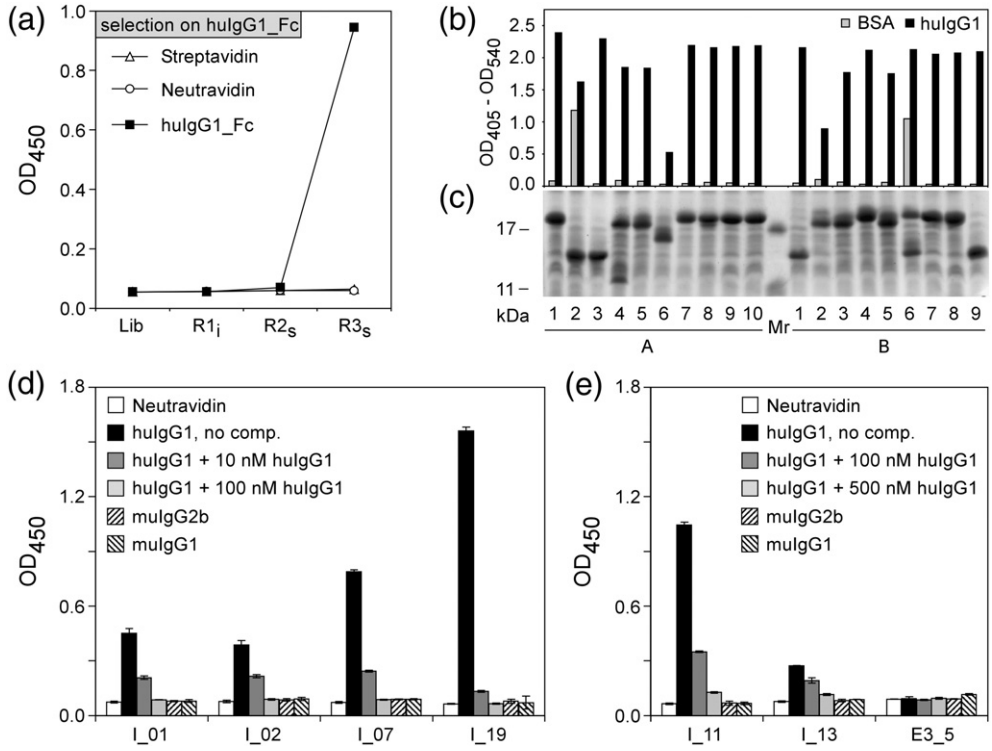

Fig. 1. Selection, screening and characterization of binders against huIgG1_Fc. (a) The enrichment for the selection performed on huIgG1_Fc was analyzed by phage ELISA. Equivalent amounts of the initial library (Lib) and pools of amplified phage particles from each selection round $R1_i$, $R2_s$ and $R3_s$ (subscript i denotes selection on immobilized target protein, subscript s denotes selection on soluble target protein) were tested for binding to immobilized streptavidin, neutravidin and immobilized huIgG1_Fc. The signal of bound phage particles, detected with an anti-M13 antibody, is shown for each sample applied. (b) ELISA screening to identify DARPins with affinity for huIgG1_Fc. *E. coli* extracts of randomly picked clones expressing soluble DARPins of selection round 3 were analyzed for binding to immobilized full-length huIgG1 and BSA (control). Positions A1 – A10 and B1 – B9 of a 96-well plate are shown. (c) SDS-PAGE analysis of the *E. coli* extracts used in (b) for ELISA screening. The mass of the SDS-PAGE protein markers is indicated in kDa. (d) ELISA with high-affinity huIgG1_Fc binders. To analyze specificity, 1 nM solutions of the purified DARPins I_01, I_02, I_07 and I_19 were tested for binding to neutravidin, huIgG1, mouse IgG1 (muIgG1) and mouse IgG2b (muIgG2b). In parallel, competition was tested by incubation of 1 nM solutions of the DARPins with 10 nM or 100 nM free huIgG1 before binding on immobilized huIgG1. (e) ELISA with low-affinity huIgG1_Fc binders and control DARPin E3_5. To analyze specificity, 5 nM solutions of the binders I_11, I_13 and control DARPin E3_5 were tested for binding on neutravidin, huIgG1, muIgG1 and muIgG2b, and in parallel, competition was tested by incubation of 5 nM solutions of the DARPins with 100 nM or 500 nM of free huIgG1 before binding on immobilized huIgG1. All full-length IgGs were immobilized directly on solid supports. The first word in the legend denotes the protein immobilized, *no comp.* denotes the absence of a competitor and + *huIgG1* denotes the presence of competitor in concentrations of 10 nM, 100 nM or 500 nM.

the well characterized, non-binding DARPin E3_5.[13] At a concentration of 10 μM, four of the six clones tested eluted at the size expected for the monomer (Fig. 2). Clone I_19 showed a second elution peak at lower elution volume, indicating some dimer formation at this concentration. I_01 eluted as a single peak but at lower elution volume (higher apparent molecular mass) than expected, indicating an increased hydrodynamic radius due to multimerization or partial unfolding. Further analysis of this binder by SEC combined with multi-angle light-scattering (SEC-MALS) revealed a clearly dimeric mass of the DARPin I_01.

Four of the clones were further analyzed by SPR (Table 3 and Supplementary Data Fig. 2) and the data obtained were evaluated with a global kinetic fit. I_19 had the highest affinity for huIgG1_Fc (K_D= 2.1 nM) and I_11 the lowest affinity (K_D= 137 nM), in good agreement with the competition ELISA experiments performed. To calculate the affinity of I_19, we assumed that the DARPin would be completely monomeric at all concentrations used for the SPR measurements (the maximal concentration of DARPin was 75 nM), and the global fit agrees very well with a 1:1 interaction (see Supplementary Data). The sequences are shown in Supplementary Data Fig. 3.

In summary, we were able to enrich a diverse set of highly specific binders with affinities in the low nanomolar range in just three rounds of selection, from our phage DARPin library.

Selection of DARPins binding to TNFα

Antibodies to TNFα have been developed for a number of therapeutic applications,[24] as has the

Table 2. Summary of the phage selection of DARPins against different target proteins

Target protein	Round of selection	No. positive clones[a]	No. different sequences[b]	Binders further analyzed in this study
huIgG1_Fc	R3	82/94	8/21	I_01/02/07/11/13/19
TNFα	R2/3[c]	102/188	29/52	T_01/02/07/08/09/16/25/27/37/40
ErbB1	R2/3[d]	97/369	1/64	E_01
ErbB1	R1 (epitope-masking)[e]	81/105	4/20	E_67/68/69
ErbB2–509	R3	83/94	13/29	9_16/26/29
ErbB2–631	R1 (epitope-masking)[e]	24/94	8/16	H_14
ErbB4	R3	55/143	5/48	B4_01/02/07/33/45
ErbB4	R1 (epitope-masking)[e]	84/94	6/20	B4_50/58
CitS[f]	R3/4	110/124	11/21	cp34_15/16[f]

[a] Ratio of the number of positive clones to the total number of clones screened by ELISA.
[b] Number of clones with different sequences confirmed by ELISA using purified DARPins to bind the target protein, identified in the total number of clones sequenced.
[c] Sum of the analysis of the second and third round of selection.
[d] Sum of the analysis of the second and third round of different selection approaches.
[e] One round of selections with epitope-masking using preselected pools as input as described in Results.
[f] Huber *et al.* [37]

soluble recombinant receptor of TNFα, Etanercept (Enbrel™, see below),[25] and binding molecules based on novel scaffolds are currently being developed to do the same. To enrich binders specific for the soluble trimeric form of TNFα (Table 1) we performed selections and screenings as described above for huIgG1_Fc using biotinylated TNFα (see Materials and Methods). Fast enrichment of phage particles displaying DARPins specific for TNFα was already observed after the second selection round. From rounds 2 and 3, 94 individual DARPins each were screened by crude extract ELISA for TNFα binding, giving 15 and 87 specific binding signals, respectively (Table 2). On the basis of the ELISA signals, 52 clones were submitted for sequencing, resulting in a highly diverse set of 29 different sequences of DARPins; interestingly, all selected DARPins were of the N3C type. Binders were expressed in the cytoplasm, purified by IMAC and further analyzed by SEC; they show high-level expression and most of them have monomeric elution behavior, similar to the results obtained for the huIgG1_Fc binders described above.

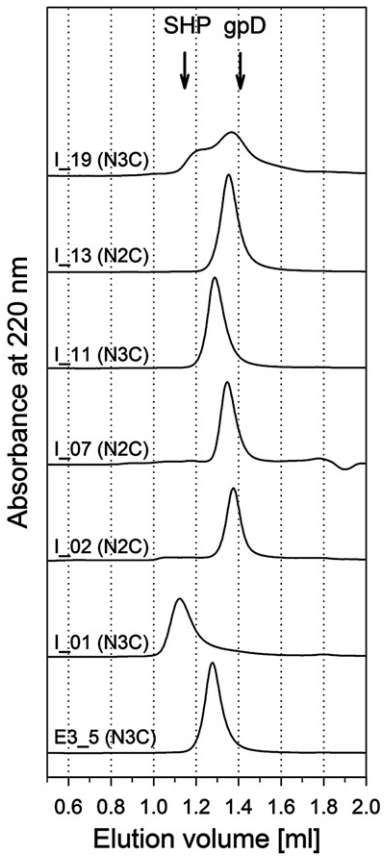

Fig. 2. Size-exclusion chromatography (SEC) of selected DARPins. The chromatograms of the six huIgG1_Fc binders and the unselected DARPin E3_5 are shown as examples. For each DARPin, the number of repeats stacked between N-Cap (N) and C-Cap (C) is given in parentheses. The molecular mass standards, phage protein D (gpD) with a mass of 17.6 kDa and phage protein SHP, a trimer with a mass of 50.2 kDa, are indicated by arrows.

We used ELISA to investigate the specificity of the selected DARPins (Fig. 3). All of the binders showed specific binding to TNFα, no interaction with neutravidin, and in the competition ELISA setup the binding to immobilized TNFα was well inhibited by preincubation of the DARPins with free TNFα. From the competition ELISA signals obtained with 10 nM free TNFα, the affinities of the binders were estimated to be in the low nanomolar range. Interestingly, the binding of all selected DARPins is inhibited almost completely by preincubation of the immobilized TNFα with Etanercept (Enbrel™, recombinant human soluble tumor necrosis factor alpha receptor fused to the Fc domain of human IgG1, Amgen, USA) (Fig. 3).

During further analysis, very much to our surprise, we discovered that the selected DARPins show specific TNFα binding that is dependent on the detergent Tween-20 that was present during the selection. We did not analyze this particular set of DARPins further, since we do not expect them to bind their target protein *in vivo*. To prevent the enrichment

Table 3. Affinities and binding kinetics of DARPins

Target protein	Clone (NxC)[a]	Oligomerization status[b]	K_D (nM)	k_{on} (10^5 $M^{-1}s^{-1}$)	k_{off} ($10^{-3}s^{-1}$)
ErbB1[c]	E_01 (N3C)	m	0.5	4.4	0.2
	E_67 (N3C)	m	7.3	0.3	0.2
	E_68 (N3C)	m/d	0.7	26	1.9
	E_69 (N4C)	m	15	0.7	1.1
ErbB2–509[c]	9_16 (N3C)	m	6.9	1.2	0.9
	9_26 (N3C)	m	1.4	0.7	0.1
	9_29 (N3C)	m	3.8	2.0	0.8
ErbB2–631[d]	H_14 (N3C)	m	0.2	4.1	0.1
ErbB4[c]	B4_01 (N4C)	m	0.1	7.3	0.06
	B4_02 (N3C)	m	0.4	1.2	0.05
	B4_50 (N4C)	m	0.3	35	1.0
	B4_58 (N5C)	m/d	9.0	16	14
IgG1_Fc[c]	I_02 (N2C)	m	41	2.8	12
	I_07 (N2C)	m	25	0.7	1.7
	I_11 (N3C)	m	140	4.9	67
	I_19 (N3C)	m/d	2.1	3.4	0.7
CitS[e]	cp34_15 (N3C)	m	5.4	–	–
	cp34_16 (N3C)	m	1.3	–	–

[a] Name of the respective clone and, in parentheses, the number of internal repeat modules (x) stacked between the N-Cap (N) and the C-Cap (C).
[b] Oligomerization status (monomer, m; dimer, d) of the DARPins determined by SEC, using globular proteins as molecular mass standards, and comparing them to other DARPins, whose oligomerization status had been verified by multi-angle light-scattering.
[c] Association (k_{on}) and dissociation (k_{off}) rate constants were measured by surface plasmon resonance, and the respective dissociation constant K_D was calculated as (k_{off}/k_{on}). Plots of the kinetic data, evaluation with a global kinetic fit and statistical error in the parameters are given in Supplementary Data Fig. 2.
[d] C. Jost and A.P., unpublished results.
[e] Dissociation constants K_D were measured by equilibrium titration (BioVeris).[37]

of such binders, Tween-20 can be omitted or replaced by other detergents in new selection approaches on TNFα.

Selection of DARPins to members of the ErbB receptor family

Antibodies to ErbB1 (EGFR) and the other members of the ErbB receptor family, notably ErbB2 (HER2), are being developed as anti-tumor therapeutics.[26] Here, we describe the selection of DARPin binders against ErbB1, ErbB2 and ErbB4. The extracellular part of all members of this family is composed of four domains (I–IV).[27] The selections on ErbB2 are described below. For both ErbB1 and ErbB4, the extracellular domains I–III of the respective receptor fused to huIgG1_Fc were used in selections (see Table 1). (The different selection approaches performed on these two target proteins are described in detail in the Supplementary Data.)

Individual DARPins were screened for specific target protein binding by crude extract ELISA as described for huIgG1_Fc. For ErbB1, 97 out of 369 and for ErbB4, 55 out of 143 screened clones gave a specific binding signal (Table 2). On the basis of ELISA signals 64 clones for ErbB1 and 48 clones for

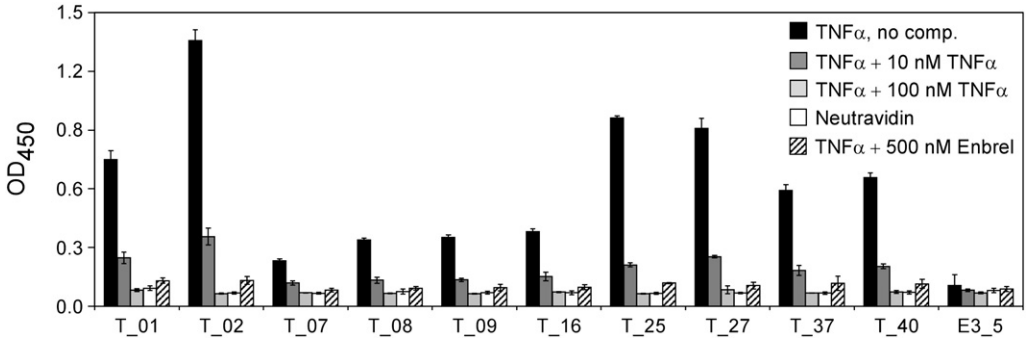

Fig. 3. ELISA of selected TNFα-binding DARPins. To analyze specificity, 5 nM solutions of the selected DARPins were tested for binding on neutravidin, immobilized TNFα and immobilized TNFα preincubated with an excess (500 nM) of Etanercept (Enbrel™, recombinant human soluble tumor necrosis factor alpha receptor fused to the Fc domain of human IgG1). In parallel, competition was tested by incubation of 5 nM solutions of the DARPins with 10 nM or 100 nM free TNFα before binding on immobilized TNFα. Only a subset of the selected TNFα binders is shown, and unselected DARPin E3_5 was used as control. The first word in the legend denotes the protein immobilized, *no comp.* denotes the absence of a competitor and + *TNFα* or + *Enbrel* denotes the presence of competitor (respective concentrations given in nM).

ErbB4 were submitted for sequencing. Surprisingly, this resulted in only one dominant ErbB1 binder (E_01) and five ErbB4 binders (B4_01, B4_02, B4_07, B4_33 and B4_45), of which B4_02 was strongly over-represented among the sequenced clones (~ 66 %).

To increase the diversity of the selected binders and to avoid dominant enrichment of these binders, we used a selection procedure termed epitope-masking.[28] For this purpose, we expressed and purified the two dominant binders (E_01 and B4_02) by IMAC and performed one round of selection on the two target proteins in solution in the presence of an only twofold excess of the respective purified binder. By this partial masking of the epitope of the dominant binder we expected to select DARPins that recognize a different epitope, but at the same time, still allow the selection of new high-affinity DARPins competing for the same epitope. Using higher concentrations of the competitor resulted in a lower diversity of the selected binders (see below). For the target proteins ErbB1 and ErbB4, equivalent amounts of amplified, partially enriched pools of phage particles after selection round 1 on immobilized target proteins (Supplementary Data Fig. 4(a) and 4(b)) and after selection round 2 on target proteins in solution (Supplementary Data Fig. 4(c) and 4(d)) were used as input. After one round of selection, input and output pools of phage particles were analyzed by phage ELISA for binding to their target protein in the absence and in the presence of a large excess (2 μM) of the respective dominant binders (Fig. 4a and b). For ErbB1, the binding signal obtained on target protein alone was much higher than the signal obtained on ErbB1 in the presence of the DARPin E_01, indicating that most of the DARPins selected indeed recognize the same or an epitope overlapping with that of DARPin E_01 (Fig. 4a). In contrast, for ErbB4, the binding signal obtained on target protein alone or in the presence of B4_02 was identical, indicating that in this case, most of the selected DARPins recognize an epitope different from that of DARPin B4_02 (Fig. 4e).

To analyze the success of the epitope-masking, individual selected DARPins were first screened for binding to their target protein by crude extract ELISA as described for huIgG1_Fc. For ErbB1, 81 of 105, and for ErbB4, 84 of 94 screened clones gave a specific binding signal (Table 2). On the basis of the ELISA signals, 20 clones of each selection were submitted for sequencing, resulting in four new binders for ErbB1 and six new binders for ErbB4. Binders were expressed in the cytoplasm of *E. coli*, purified and analyzed further by SEC. They show high-level expression and most of them have monomeric elution behavior, similar to the results obtained for the huIgG1_Fc binders described above. All of the clones bound specifically to the respective target protein, as shown by competition ELISA, but only those ErbB1 binders (E_67, E_68, E_69) and ErbB4 binders (B4_50, B4_58) were analyzed in detail whose ELISA signals could be inhibited to less than 50% with 10 nM (50 nM for E_69) ErbB1 or ErbB4 as competitor, suggesting a high affinity (cf Fig. 1d).

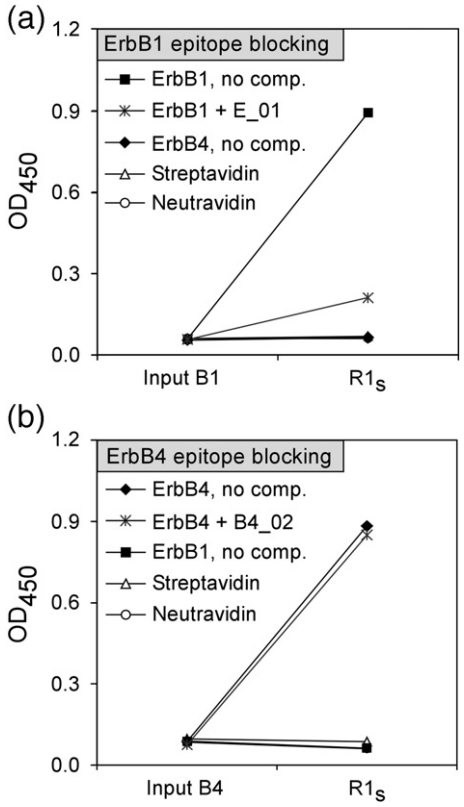

Fig. 4. Equivalent amounts of the input and output phage particles from one round of epitope-masking performed on ErbB1 (by adding soluble DARPin E_01) and on ErbB4 (by adding soluble DARPin B4_02) were analyzed by phage ELISA as described for Fig. 1. Additionally, binding was tested on the respective target protein preincubated with an excess of the respective DARPin (*ErbB1 + E_01* or *ErbB4 + B4_02*). Subscript s denotes selection on target protein in solution. The first word in the legend denotes the protein coated, *no comp.* denotes the absence of a competitor and + *E_01* or + *B4_02* denotes the presence of 2 μM DARPin competitor.

This was confirmed by SPR analysis (see below). (The sequences are shown in Supplementary Data Fig. 3.)

Clones selected by epitope masking show diverse specificities

Binding and epitope localization of the clones selected by epitope masking were tested by ELISA. All high-affinity ErbB1 and ErbB4 binders were tested for interaction with the respective biotinylated target protein bound to immobilized neutravidin, or the nonbiotinylated target protein bound to the immobilized DARPins (E_01 or B4_02, respectively) used for epitope-masking (Fig. 5a and b). In the selection on ErbB1 using an only twofold excess of E_01, three new high-affinity clones were selected. E_69 recognized an epitope not competing with E_01, whereas the other two binders, E_67 and E_68, must recognize a competing epitope, as they could not bind to their target, immobilized over E_01.

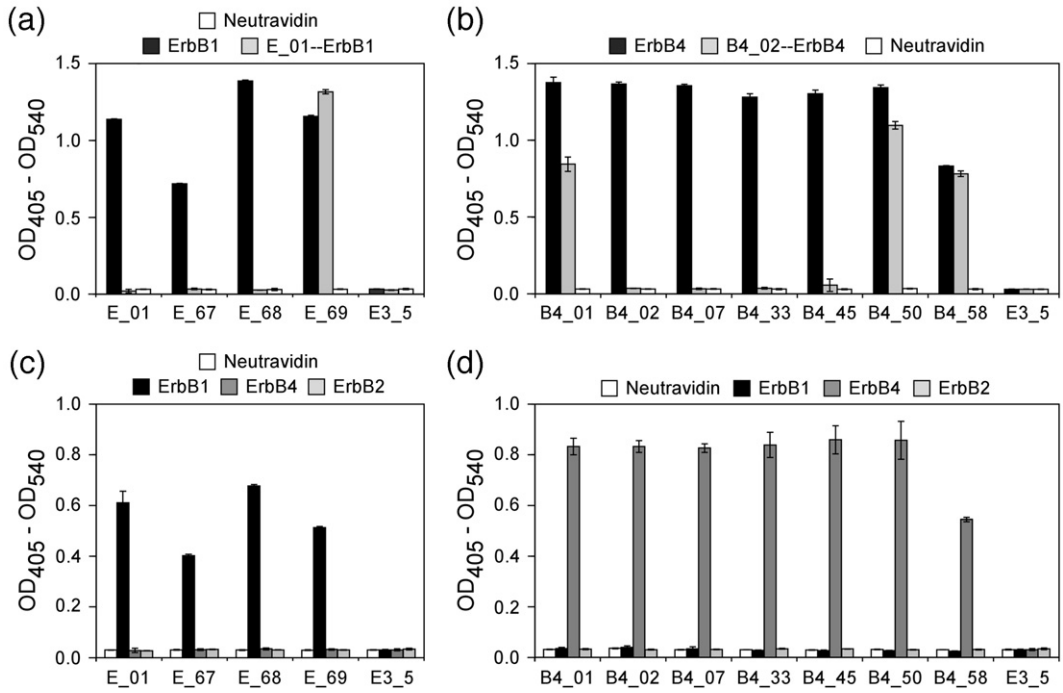

Fig. 5. ELISA of selected ErbB1 and ErbB4 binding DARPins. A 50 nM solution of the selected ErbB1 (a) and ErbB4 (b) binders was tested for interaction with immobilized target protein (ErbB1 or ErbB4, respectively), target protein bound to immobilized DARPin used for epitope-masking during the selection round (E_01—ErbB1 or B4_02—ErbB4, respectively), and neutravidin. (c, d) Specificity was tested by applying a 50 nM solution of the selected ErbB1 binders (c) and ErbB4 binders (d) on immobilized ErbB-receptors (ErbB1, ErbB2-631 and ErbB4) and neutravidin. Unselected DARPin E3_5 was used as control in all experiments. ErbB1 binders E_67, E_68 and E_69 and ErbB4 binders B4_50 and B4_58 were selected by epitope masking.

When performing the same epitope-masking selection in the presence of a much higher excess (20-fold) of the masking DARPin (E_01), clone E_69 was very dominant. This finding shows that somewhat higher levels of diversity can be obtained by only partially masking the dominant epitope than by completely blocking it. In contrast, for ErbB4, one of the clones (B4_01) selected on ErbB4 in solution and both of the clones selected by epitope-masking recognized an epitope other than B4_02. None of the binders showed cross-reactivity when analyzed in ELISA experiments for binding to other receptors of the ErbB-family (ErbB1, ErbB2 and ErbB4), which share about 50% sequence identity and have highly conserved structures (Fig. 5b and c). To estimate affinity, competition experiments with ErbB1 and ErbB4 were performed as described for huIgG1_Fc, and ELISA binding signals were inhibited to less than 50% by 10 nM competitor (50 nM for E_69), suggesting low nanomolar affinity (data not shown). This was confirmed by SPR analysis (see below).

Four well-behaved clones for each of the target proteins were further analyzed by SPR (Table 3 and Supplementary Data Fig. 2) and the data were evaluated with a global kinetic fit. Five of the measured binders showed affinities in the picomolar range, and the other binders were all below 15 nM.

In conclusion, dominant epitopes on a target protein may hinder the direct selection of diverse binders. This "hidden" diversity present in the library can be made accessible by masking the dominant epitope during the selection cycle. Strong masking, however, can lead to a new focusing of the library.

Epitope removal allows selection of more diverse DARPins

To enrich binders against ErbB2 containing all four domains (I – IV) of the extracellular part of the receptor and thus comprising the first 631 amino acid residues of the mature protein (termed ErbB2-631, see Table 1), we performed three initial rounds of selection on the target protein in solution. Good enrichment of phage particles displaying DARPins binding to ErbB2-631 but not to ErbB2-509 (see Table 1), which comprises only the first 509 amino acid residues of the mature protein and therefore lacks domain IV, was observed after the third selection round, as shown by phage ELISA (Fig. 6a). This is in good agreement with previously performed ribosome display selections, where all selected high-affinity binders recognized domain IV, which seems to contain one or more dominant epitopes of that protein.[20]

We used two strategies to obtain binders that recognize domains I – III of the target protein. First, we performed selection directly on ErbB2-509. Surprisingly, at room temperature, all of the selection approaches on ErbB2-509, independent of whether the selection had been performed on immobilized

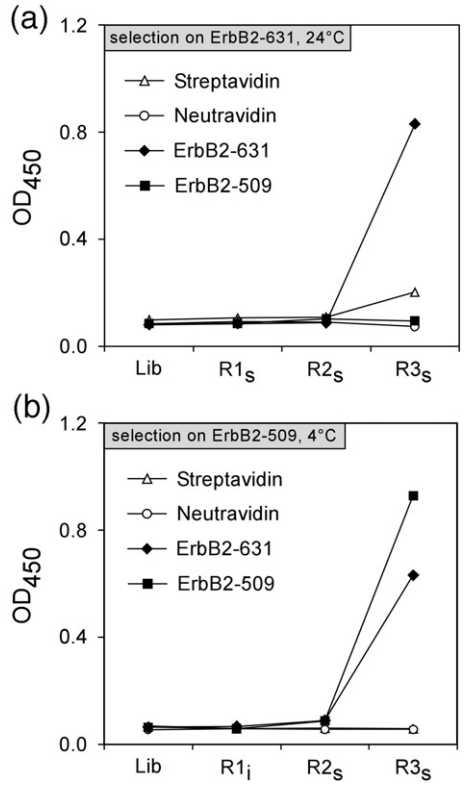

Fig. 6. The enrichment for the selections performed on ErbB2-631 in solution at room temperature and on ErbB2-509 at 4 °C were analyzed by phage ELISA. Equivalent amounts of the initial library (Lib) and pools of amplified phage particles from each selection round (R1, R2, and R3) were tested for binding to immobilized streptavidin, neutravidin and immobilized ErbB2-509 and ErbB2-631. The signal of bound phage particles, detected with an anti-M13 antibody, is shown for each sample applied. For each round, the subscript denotes if the selection was performed on immobilized target protein (i) or on target protein in solution (s).

target protein or on target protein in solution, failed to yield an enrichment of target-specific DARPins. We reasoned that this might be due to a lower stability or higher flexibility of the truncated version of the receptor at room temperature and therefore performed further selections at 4 °C. Indeed, after three rounds of selection on ErbB2-509 by using the optimized selection protocol at 4 °C, target-specific binding of the enriched pools was observed in phage ELISA (Fig. 6b). For the pool of round 3, specific binding to both target proteins ErbB2-631 and ErbB2-509 was observed, suggesting that domains I–III are recognized, and that target stability is indeed a very important parameter for optimal selections.

After round 3, 94 individual DARPins were screened by crude extract ELISA for binding to ErbB2-509, resulting in 83 specific binding signals (Table 2). On the basis of the ELISA signals, 29 binders were sequenced, resulting in a diverse set of 13 different sequences of DARPins. Binders were expressed in the cytoplasm, purified and analyzed further by SEC; they showed high-level expression and most of them have monomeric elution behavior in SEC, similar to the results obtained for the huIgG1_Fc binders described above.

ELISA experiments showed high specificity (no cross-reactivity with ErbB1 and ErbB4), and inhibition of ELISA signals to less than 50% was observed for more than half of the clones using 10 nM ErbB2 as competitor, suggesting nanomolar affinity (cf Fig. 1d). Three of the binders (9_16, 9_26, and 9_29) were analyzed further by SPR showing affinities in the low nanomolar range (Table 3). (The sequences are shown in Supplementary Data Fig. 3.)

The results obtained indicate that, similar to the epitope masking strategy described above, this strategy of removing an epitope by deleting a domain makes the hidden diversity of the library accessible for selection. It shows also that attention must be paid to the conformational integrity of the target protein, in this case by lowering the temperature during selection.

Additionally, as described for ErbB1 and ErbB4, we partially masked one of the epitopes (see above) on domain IV by adding a twofold excess of the high-affinity DARPin H10-2-G3 in one round of selection on ErbB2-631.[29] The amplified, partially enriched pool of phage particles after two rounds of selection on the target protein in solution (Fig. 6a) was used as input. Screening of 94 clones by crude extract ELISA resulted in 24 specific binding signals. On the basis of the ELISA signals, 16 binders were sequenced, resulting in eight different sequences, of which four had been found in the selection on ErbB2_509. Of the three new binders, two recognized ErbB2-509 as well as ErbB2_631 (domain I–III binders) and one (H_14) recognizes only ErbB2_631 (domain IV binder). Due to its exceptionally high binding signal, binder H_14 was analyzed further by SPR and the data were evaluated with a global kinetic fit showing an affinity in the picomolar range (Table 3) (C. Jost and A.P., unpublished results).

Discussion

Large functional DARPin library obtained by SRP phage display

The quality of a combinatorial library of binding proteins can be functionally described by the ability to isolate high-affinity binders against a broad set of target proteins. Three major factors contribute to successful selections from such a library. First, the library design, which encompasses the scaffold used as well as the diversification strategy employed. Second, the theoretical diversity of the combinatorial DNA library, combined with the library quality, i.e., the percentage of clones that actually have sequences as specified in the design. Finally, the display technology chosen, which should ensure that the members of the library are presented in a functional form, allowing for efficient selection. Together, these

factors determine the functional diversity of the library that is accessible for the selection of binding molecules by the technology employed.

We have developed SRP phage display that allows the efficient display of fast-folding and stable proteins such as DARPins that are virtually refractory to conventional phage display that uses Sec-dependent translocation of the POI into the periplasm.[21] We generated our phage DARPin library using the previously described combinatorial N3C DARPin library[13,16] as template and a phagemid containing an SRP-dependent signal sequence.[21] Analysis of single unselected members of this new library revealed that all clones that are correct at the sequence level are displayed on the phage particles at levels comparable to the display yield obtained for a well-expressed single-chain Fv (scFv) antibody fragment using a conventional phagemid with a Sec-dependent signal sequence. We thus obtained a functional library diversity of at least 1×10^{10} which is comparable to the size reported for the largest phage libraries displaying non-immunoglobulin domains[30] and comparable to the size of large phage libraries displaying antibody fragments.[8]

The functional part of the phage DARPin library is composed of N1C (4%), N2C (27%) and N3C (69%) DARPins, as determined from the sequencing and display analysis of unselected clones. The constructs shorter than the N3C are probably side-products of the PCR of the initial combinatorial N3C library necessary during construction; this is also observed regularly when doing ribosome display selections and results from the repetitive nature of DARPins. Interestingly, we even selected N4C and N5C DARPins (Table 3). Since no repeat duplication or deletion that would indicate *in vivo* recombination of the DARPin scaffold was ever observed, we expect these longer constructs, just as the shorter ones, to be already present at a low level in the initial library. Since the selection for binding works well, as demonstrated by the successful selection against diverse targets, the occurrence of DARPins with a different number of repeats, (presumably) already in the initial library, can be seen as a source of additional diversity. A larger contiguous patch of randomized residues in the DARPins with more than three randomized repeats may allow binding to additional epitopes, where contacts by residues in the capping repeats might not have been favorable or where a larger binding surface is needed. An accumulation of these longer constructs during selections with epitope masking supports this theory.

In just two or three phage display selection rounds we were able to select high-affinity binders against a broad range of target proteins in all cases attempted so far (Table 2). This validates the functional library diversity and the optimized diversity design of the DARPin scaffold, and that SRP phage display is robust to select specific binders from this library. Good performance of SRP phage display was recently reported for the display of a library based on the fibronectin type III domain.[31] We suppose that SPR phage display could increase the functional diversity of many other libraries, especially those expected to be composed of similarly stable and fast-folding scaffolds or those containing members with widely different folding rates and stabilities, such as expected, for example, in cDNA libraries.

It should be noted that for SRP phage display, as reported for conventional phage display,[32] it might be necessary to optimize the phagemid in combination with the scaffold used, in order to tune the expression level to achieve efficient but still monovalent display of the POI.

Selected DARPins have favorable biophysical properties

The selected DARPins retain the favorable biophysical properties of the initial library members.[13] They can be expressed in large amounts in soluble form in the cytoplasm of *E. coli* (Fig. 1b), and routinely more than 100 mg of protein is purified from 1 l of shake-flask culture. Of the 84 clones analyzed by SEC, 46 (55 %) show a single peak at the elution volume expected for the monomer (Fig. 2) and another ten (12%) show a single peak at a greater elution volume, indicating some interaction with the column material; nevertheless, they were confirmed to be monomeric by SEC-MALS measurement. The other DARPins are presumably a mixture of monomer and dimer or elute at higher apparent molecular mass, indicating oligomerization or partial unfolding, as a consequence of some particular features of the sequence of a given library member. Because of the diversity of clones isolated for each target, purely monomeric tight-binding DARPins have always been found.

The selected clones specifically recognize the target protein that they were selected on and do not cross-react with other proteins tested, as shown by ELISA (Fig. 5c and d). These ELISAs were always done with recloned non-fused protein, and thus independent of being fused to the phage coat protein 3 or being displayed on phage particles.

DARPins with picomolar affinities were selected on a regular basis

The highest affinities of the selected binders are in the sub-nanomolar range (Table 3) and thus as good as the affinities obtained when performing ribosome display selections with the identical initial DARPin library.[16–18] There are several factors that may contribute to this, at first surprising, observation. First, the functional size of the present phage library is very large, and is expected to cover the diversity of about 10^{10} members present in the initial ribosome display library that was used as template.[16] Therefore, the same input library is sampled in ribosome display and phage display; however, in ribosome display, mutations continuously increase this diversity during the selection rounds. Second, a growing experience in designing selection experiments, as summarized in the optimized selection protocol, may have improved the quality of the selected

clones. When comparing the performance of phage display and ribosome display, often no significant difference was found at the levels of the *best* binders, however, the number of different binders with such characteristics tends to be larger in ribosome display. We speculate that in early rounds, low-affinity binders may acquire point mutations in ribosome display and thus survive later selection rounds with greater stringency, adding to diversity.

From the comparison of affinities obtained from various antibody libraries, it is widely accepted that the affinities of the selected antibodies correlate with the size of the library.[8] From phage antibody fragment libraries containing $10^7 - 10^9$ members, binders with affinities of up to 10 nM, and for libraries with over 5×10^9 members, binders with sub-nanomolar affinities have been selected.[8] From our phage DARPin library containing about 10^{10} functional members we obtained against all target proteins multiple DARPins with affinities in the low nanomolar or, in most cases, even the sub-nanomolar range (Table 3). These affinities compare very favorably with those obtained for synthetic antibody repertoires of similar or even larger size.[9–11,33] Our very promising results can be attributed to the optimized diversity design of the DARPin library as well as the high functional display rate of the SRP phage display system. This will make affinity maturation unnecessary for most applications.

Broadening diversity of selected DARPins by epitope masking or removal

One frequently observed phenomenon in phage display when performing selection experiments on certain target proteins is low diversity of the selected binders, even when using large libraries.[9,34–36] There are many reasons why a certain binder might be selected with preference and dominate the selected pool: for example, (i) the binder has much higher affinity than the other binders; (ii) the binder is displayed more efficiently than the other binders, (iii) the target protein contains a preferred binding site on its surface;[35] or (iv) there are only limited epitopes on the target protein accessible to potential binders, e.g. by extensive glycosylation. When performing selections on the receptors of the ErbB family we obtained binders with low diversity (i.e. focused on a dominant epitope), suggesting that some epitopes in this family are very well suited for the DARPin binding interface, such that they dominated the selection. The dominant DARPin E_01 was also shown to be displayed at slightly higher levels than other library members (data not shown), and both E_01 and B4_02 have very high affinities, indicating that also the above-mentioned points (i) and (ii) can influence the selection of DARPins by phage display. Note that a much broader diversity was obtained for other targets (TNFα, huIgG1_Fc, CitS[37] and others, data not shown). We tested several strategies to increase the diversity of the selected DARPins and to broaden the range of selected specificities.

For ErbB1, where essentially only one binder was found in the initial selections, and for ErbB4, where one binder was very dominant (Table 2), we performed one round of selection on the target protein in the presence of a twofold excess of the purified dominant binder using pools of phage particles partially enriched on the target protein as input. This strategy of epitope-masking was described recently for antibodies.[38,39] In both cases this approach resulted in increased diversity (i.e., binding to different epitopes) of the binders (Fig. 6). For ErbB1, two of the three clones analyzed further still bound to the same or a competing epitope as the binder used for epitope-masking. This is presumably due to the low excess, only twofold, of competitor used, leading to only a partial masking of the epitope, and thus making it possible to still find binders to the same epitope.

For the full-length ectodomain of ErbB2 (ErbB2-631), the selection yielded a pool of binders that recognize this full length ectodomain but not a truncated construct lacking domain IV (Fig. 6a). Therefore, we performed selections on the truncated version. Probably due to the lower stability of ErbB2-509, selections on this target protein were successful only at low temperature. They yielded a pool of binders recognizing ErbB2-631 as well as ErbB2-509 (Fig. 6b). Therefore, both strategies, epitope-masking and epitope removal, were applied successfully, and combining such binders recognizing different epitopes would give the possibility of constructing multivalent binders for a given target protein.

Conclusions

We have validated our phage DARPin library by successfully selecting well-behaved and high-affinity binders against a broad range of target proteins. The SRP phage display used in this work has thus proven to be a powerful method to display libraries of fast-folding and stable DARPins that would be refractory to conventional phage display using the Sec translocation pathway. We envision that this novel phage display method may be beneficial for other libraries containing stable and fast-folding proteins. The addition of phage display to the selection repertoire for the generation of specific DARPins further expands the application range of this novel class of binding molecules.

Materials and methods

Materials

All chemicals were of the highest quality available and were purchased from Fluka (Switzerland) unless stated otherwise. Oligonucleotides were from Microsynth (Switzerland). Vent DNA polymerase, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas (Lithuania). Helper phage VCS M13 was from Stratagene (USA). All cloning and phage

amplification was performed in *E. coli* XL1-Blue from Stratagene (USA).

Molecular biology

Unless stated otherwise, all molecular biology methods were performed according to standard protocols.[40]

Phagemid and expression vectors

The phagemid pPDV1 (Supplementary Data Fig. 1) is related to pDST23,[21] and comprises the following features: (i) the signal sequence of *E. coli* DsbA (DsbAss) ensures efficient SRP phage display; (ii) sequences for display are inserted into the multiple cloning site (MCS) between the DsbAss and the coding sequence of the C-terminal domain (amino acids 250–406) of protein 3 (p3) of filamentous phage M13; (iii) the fusion protein (p3 fusion) is under the control of a *lac* promoter/operator element and the *lacI*^{q} gene provides high levels of the lac repressor in *cis*; (iv) an amber stop codon (TAG) is interposed between the displayed sequence and the C-terminal domain of p3; (v) the fusion protein contains a Flag-tag; (vi) the vector carries an f1 origin of replication to permit production of virions using an appropriate helper phage, such as VCS M13; and (vii) the vector has a plasmid origin (ColE1 *ori*) and an antibiotic-resistance marker (the gene for chloramphenicol acetyl transferase (*cat*) providing resistance to cam) to allow propagation as a plasmid in *E. coli.*

The vector pDST67 was used for high-level, soluble cytoplasmic expression of the selected DARPins containing an N-terminal MRGS(H)$_6$ tag. pDST67 is a derivative of pQE30 (QIAgen) in which the single amber stop codon (TAG) after the HindIII restriction site has been replaced by a double stop codon (TAA TGA), avoiding the formation of a read-through product when using suppressor *E. coli* strains such as XL1-Blue. pDST67 was generated via PCR cloning using the oligonucleotides oDST29 5′-GGCCAAGCTTAATTAATGACTGAGCTTGGACTCCTG-3′ and oDST30 5′-AAAGCCAAGCTAGCTTGGATTCTC-3′ and pQE30 as template. The resulting PCR product was HindIII/NheI-digested and ligated into the identically treated and dephosphorylated pQE30 vector. Coding sequences of the selected DARPins were cloned into pDST67 via BamHI/HindIII, yielding plasmids termed pDST67_(DARPin Name). Protein expression and purification are described below.

The vector pAT223 (GenBank accession number AY327138) was used for the expression of fusion proteins with an N-terminal Avi-tag for *in vivo* biotinylation, followed by phage lambda protein D (pD) as fusion protein, followed by a His$_6$ tag for IMAC purification and then the protein of interest, e.g. a DARPin to be biotinylated and immobilized. Coding sequences of the selected DARPins E_01 and B4_02 were cloned into pAT223 via BamHI/HindIII, yielding pDST126 and pDST127, respectively. Protein production, purification and confirmation of biotinylation were as described.[17]

Library construction

The N3C DARPin library in the ribosome display format[13] was PCR amplified using primers introducing a BamHI and HindIII site (EWT3 and WTC4),[16] enabling subsequent subcloning into phagemid pPDV1. A total of 200 PCR reactions of 50 μl yielded about 600 μg of purified PCR product, which was ligated after restriction digest of the components using BamHI and HindIII into pPDV1. About 300 μg of ligated and purified DNA was first transformed by electroporation into MP1 cells (*E. coli* strain MC1061)[41] containing the F episome transferred from *E. coli* XL1-Blue cells) as these cells can be transformed very efficiently,[42] yielding 3.6×10^{10} independent colonies. From these cells, the plasmid DNA was isolated and re-transformed into *E. coli* XL1-Blue cells, for subsequent phage production, yielding 2.6×10^{10} independent colonies. The transformed XL1-Blue cells were infected with helper phage VCS M13, and IPTG was subsequently added to the culture to induce the expression and thus the display of the DARPins, thereby producing the phage particles of the phage DARPin library. The DARPin-displaying phage particles were precipitated with PEG from the culture supernatant, resuspended in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 10% (v/v) glycerol, and stored at -80 °C, where they were stable for at least 1 year.

Library characterization

To determine the percentage of transformed clones encoding functional DARPins, 55 randomly picked clones were sequenced using standard techniques. To analyze the fraction of DARPin-p3 fusion proteins displayed, phage particles produced form the 55 sequenced clones were analyzed by Western blot as described,[21] using the anti-FLAG antibody M1 (Sigma, USA) for detection. Phage particles displaying an scFv, known to be well displayed using a conventional phagemid, were used as a reference; phage particles produced form phagemid pDST24.[21] To quantify the percentage of DARPins showing high-level, soluble cytoplasmic expression, a pool of DARPin inserts from the phage library was subcloned into pDST67 via BamHI/HindIII. Small-scale expression and SDS-PAGE (15% (w/v) polyacrylamide gel) analysis of 57 randomly picked clones, which were not sequenced, was performed.

Target proteins

Purified target proteins were used for all experiments and are described in detail in Table 1. ErbB receptors were kindly provided by Dr Tim Adams and coworkers (CSIRO, Melbourne, Australia). The Fc domain of human IgG1 was purchased from R&D Systems, UK. For immobilization, aliquots of these target proteins (200–600 μg) were chemically biotinylated using EZ-Link Sulfo-NHS-SS-Biotin (Pierce, USA). Due to the size difference of the target proteins, a variable molar excess of the biotinylating reagent relative to the target protein was used (fourfold for huIgG1_Fc, 9-fold for ErbB1, 6-fold for ErbB2-509, 6-fold for ErbB2-631 and 9-fold for ErbB4). Reaction conditions were used according to the supplier's manual (Pierce, USA). Successful biotinylation was confirmed by ELISA and Western blot experiments, but the extent of biotinylation was not quantified.

Non-biotinylated and biotinylated TNFα, shown to be biologically active, was expressed and purified from *E. coli* (C. Zahnd and A.P., unpublished results). In brief, human TNFα was amplified using the primers rTNFstopHind 5′-GATGAGAAGCTTTCATTACAGGGCAATGATCCCAAAG-3′ and fTNFa 5′-CACCACCATGGCTGTCAGATCATCTTCTCGAAC-3′. The resulting PCR product was NcoI and HindIII-digested and ligated into the identically treated and dephosphorylated vector pAT222 (GenBank accession number AY327137), yielding the construct with an N-terminal Avi-tag[23] used for the expression of bio-

tinylated TNFα and into the vector pQE60 (QIAgen), yielding the construct with no tag used for the expression of non-biotinylated TNFα. For biotinylation, the biotin-ligase BirA of *E. coli* was cotransformed and co-expressed with the Avi-tagged construct in *E. coli* XL1-Blue to yield *in vivo* biotinylated TNFα as described[23]. The biotinylated TNFα was purified *via* a monomeric avidin column. To allow intramolecular disulfide bond formation, the soluble protein was dialyzed against TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 1 mM reduced glutathione (GSH) and 0.2 mM oxidized glutathione (GSSG). The trimeric species was obtained by preparative gel-filtration chromatography in using TBS as the running buffer. The TNFα construct with no tag was expressed in *E. coli* XL1-Blue, purified via a cation-exchange column using washing buffer (100 mM MES, pH 5.8) with a NaCl gradient from 150 mM – 1 M, followed by a dialysis step against the GSH/GSSG buffer mentioned above and subsequent preparative gel-filtration chromatography. The biological activity of both constructs was shown by their cytolytic activity on L929 mouse fibroblast cells (ATTC, USA) and compared to commercial TNFα. Since the specific activity was the same within experimental error, and since disulfide-deficient mutants are known to show reduced biological activity,[43,44] we assume that the disulfides have been formed correctly.

Immobilization of target proteins

Target proteins had to be immobilized for selection and ELISA experiments. To avoid partial protein denaturation of the target proteins that may result from direct immobilization on solid plastic (i.e. polystyrene) surfaces, biotinylated target proteins were bound to neutravidin or streptavidin, which had been immobilized directly on a solid plastic surface, as follows: neutravidin (66 nM, 100 μl/well, Pierce, USA) or streptavidin (66 nM, 100 μl/well Sigma, Switzerland) in PBS was immobilized on MaxiSorp plates (Nunc, Denmark) by incubation at 4 °C overnight. The wells were blocked with 300 μl of PBSTB (PBS containing 0.1% Tween-20, 0.2% BSA) for 1 h at room temperature. Binding of the biotinylated target proteins (100 μl, ~ 100 nM for selection and ~20 nM for ELISA) in PBSTB was allowed to occur for 1 h at 4 °C.

For the first selection round on immobilized target protein, requiring larger volumes, neutravidin (66 nM, 4 ml/tube) in PBS was immobilized on MaxiSorp Immunotubes (Nunc, Denmark) by incubation at 4 °C overnight. The tubes were blocked with 4 ml of PBSTB for 1 h at room temperature. Binding of the biotinylated target proteins (4 ml, ~100 nM) in PBSTB was allowed to occur for 1 h at 4 °C.

For selection on immobilized target protein, neutravidin and streptavidin were used alternately in selection rounds to avoid selection of binders against these proteins.

Selection of phage DARPins

Unless stated otherwise, all steps of the phage display selection were done at room temperature. Selection rounds were performed either on biotinylated target protein in solution with subsequent capturing on streptavidin-coated magnetic beads (referred to as: target protein in solution) or on biotinylated target protein bound to neutravidin or streptavidin, which had been immobilized directly on a solid plastic surface (referred to as immobilized target protein), as described below. Very good results were obtained when performing the first selection round of selection on immobilized target protein, presumably because of the greater efficiency of capturing binders (especially important in the first round), followed by further rounds on target protein in solution, presumably because of the lower enrichment of background binders (for the optimized selection protocol, see Supplementary Data). Protocols were adapted from Ref. 45.

Selection on target proteins in solution

When the first selection cycle was done in solution, about 2.5×10^{13} phage particles of the phage DARPin library were incubated for 1 h with 100 nM biotinylated target protein in 2 ml of PBSTB for the first round of selection. In subsequent selection rounds, about 10^{12} phage particles were used (see below). The phage–antigen complexes were then captured on 100 μl streptavidin-coated paramagnetic beads (10 mg/ml, Dynabeads MyOne Streptavidin T1, Dynal) for 20 min. After washing the beads eight times with PBST (PBS, 0.1% Tween-20) the phage particles were eluted with 200 μl of 100 mM triethylamine (Et_3N, pH not adjusted) for 6 min, followed by 200 μl of 100 mM glycine-HCl, pH 2, for 10 min. Eluates were neutralized with 100 μl of 1 M Tris–HCl, pH 7, or 18 μl of 2 M Tris-base, respectively, combined and used to infect 5 ml of exponentially growing *E. coli* XL1-Blue cells. After shaking for 1 h at 37 °C, cells were expanded into 50 ml of fresh 2YT medium (5 g of NaCl, 10 g of yeast extract, and 16 g of tryptone per liter) containing 10 μg/ml cam and incubated at 37 °C with shaking. After a maximum of 5 h (shorter times if $A_{600}=0.5$ was reached earlier), isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and 15 min later the phage library was rescued by infection with VCS M13 helper phage at 10^{10} pfu (plaque-forming units) per ml (multiplicity of infection ~ 20). Cells were grown overnight at 37 °C without the addition of kanamycin. Cells were removed by centrifugation (5600*g*, 4 °C, 10 min) and 40 ml of the culture supernatant was incubated on ice for 1 h with one-fourth volume of ice-cold PEG/NaCl solution (20 % (w/v) polyethyleneglycol (PEG) 6000, 2.5 M NaCl). The precipitated phage particles were then collected by centrifugation (5600*g*, 4 °C, 15 min) and redissolved in 2 ml of PBS and used for the second round of selection.

For the subsequent selection rounds, about 10^{12} of the amplified phage particles were used as input and incubated with 100 μl of streptavidin-coated paramagnetic beads for 1 h to remove unspecific and streptavidin-binding phage particles. After removing the beads, phage particles were incubated for 1 h with 100 nM biotinylated target protein, complexes were captured on fresh beads, beads were washed 12 times with PBST, phages eluted with 400 μl of 100 mM glycine-HCl, pH 2, for 10 min, the eluate neutralized with 36 μl of 2 M Tris-base and phage particles amplified and purified as described above.

After three rounds, enrichment of phage particles displaying DARPins binding specifically to the target protein was monitored by phage ELISA. About 5×10^{10} phage particles (estimated spectrophotometically[46]) of the initial library and the amplified pools of each selection round were pipetted into wells with and without immobilized target protein and kept at room temperature for 2 h. After washing the wells four times with 300 μl of PBST, bound phage particles were detected with mouse anti-M13 antibody horseradish peroxidase conjugate (Amersham Pharmacia Biotech, UK) and soluble BM Blue peroxidase (POD) substrate (Roche Diagnostics, Germany).

Selection on immobilized target proteins

For the first selection cycle about 3.5×10^{13} phage particles of the phage DARPin library were added to an immunotube containing the immobilized target protein (biotinylated target protein bound to neutravidin, which had been immobilized directly on the solid plastic surface) and incubated with rotation for 2 h. After rinsing the tube ten times with PBST, the phage particles were eluted with 500 μl of 100 mM Et_3N (pH not adjusted) for 6 min, followed by 500 μl of 100 mM glycine-HCl, pH 2, for 10 min. Eluates were neutralized with 250 μl of 1 M Tris–HCl, pH 7, or 45 μl of 2 M Tris-base, respectively, combined and used to infect 13 ml of exponentially growing *E. coli* XL1-Blue cells. After shaking for 1 h at 37 °C cells were expanded into 130 ml of fresh 2YT medium containing 10 μg/ml cam and incubated at 37 °C with shaking. Phage amplification and precipitation was done as described above.

In the subsequent selection rounds about 10^{12} of the amplified phage particles were first incubated in a blocked immunotube (coated either with neutravidin or streptavidin used for immobilization of the target protein in the previous round of selection and BSA) for 1 h to remove neutravidin, streptavidin or unspecific binding phage particles. For the binding selection, the phage particles were incubated for 1 h in four wells containing the immobilized biotinylated target protein (directly coated neutravidin or streptavidin were used alternately in subsequent selection rounds). The wells were washed 12 times with PBST, phages eluted from each well with 100 μl of 100 mM glycine–HCl, pH 2, for 10 min, the combined eluates neutralized with 36 μl of 2 M Tris base and phage particles amplified and purified as described above. After three rounds, enrichment was determined by phage ELISA as described above.

Selection with epitope-masking

The respective DARPins used for the epitope masking were expressed in 50 ml shaking flask cultures and purified by IMAC as described.[13] Pools of phage particles already partially enriched for binders against the respective target protein (one or two rounds of selection on the target protein alone as described above) were used as input. Biotinylated target protein (100 nM) was pre-incubated for 1 h with the respective DARPin (200 nM or 2 μM) before performing a standard selection round on soluble target protein as described above. After one round of selection with epitope-masking, amplified phage particles were analyzed by phage ELISA. Binding to immobilized target protein alone and in the presence of an excess of the respective DARPin (2 μM) used for epitope-masking was done as described above.

Screening of single clones

From the selected phage pools showing specific binding to the respective target protein, the DNA fragments encoding the DARPin inserts were subcloned into the expression vector pDST67 via BamHI/HindIII.

Single selected DARPins were expressed in 96-deep well plates. In brief, 1.2 ml of 2YT medium containing 1 % (w/v) glucose and 50 μg/ml ampicillin was inoculated with a single, randomly picked colony of *E. coli* XL1-Blue harboring pDST67 encoding one of the respective selected DARPins and incubated overnight at 37 °C with shaking. Fresh 2YT medium (1.1 ml) containing 50 μg/ml ampicillin was inoculated with 100 μl of the overnight culture. After incubation at 37 °C for 1–2 h with shaking, 100 μl of 6.5 mM IPTG in 2YT was added to each well and protein expression was continued for 4 h. Cells were harvested by centrifugation, resuspended in 50 μl of B-PER II (Pierce, USA) by vigorous shaking for 20 min at room temperature. Then, 950 μl of PBSTB was added and cell debris was removed from the crude extract by centrifugation.

For the ELISA, 10–100 μl of the above crude extracts was applied to wells with or without immobilized target protein and incubated at room temperature for 1 h. After washing the wells three times with 300 μl of PBSTB, wells were incubated with an anti RGS-His antibody (QIAgen, Germany) (1:5,000 in PBSTB, 1 h at room temperature), as primary antibody. A goat anti–mouse IgG alkaline phosphatase conjugate (Sigma, USA) (1:10,000 in PBSTB, 1 h at room temperature) was used as secondary antibody. The bound DARPins were detected with the substrate di-sodium 4-nitrophenyl phosphate (pNPP, 3 mM) (Fluka, Switzerland) in buffer containing 50 mM $NaHCO_3$ and 50 mM $MgCl_2$ (Fig. 1b).

The 96-well purification of DARPins

Selected DARPins identified as binders were expressed in 96-deep well plates as described above. Eight plates containing fresh 2YT medium were inoculated in parallel with the overnight culture, resulting in a total expression culture volume of about 10 ml for each clone. Cells were lysed as described above, the 400 μl B-Per II lysates for each clone were pooled and 400 μl of TBS_{500} (50 mM Tris–HCl, pH 8, 500 mM NaCl), containing 20% (v/v) glycerol and 40 mM imidazole, were added. After centrifugation the cleared lysates were used for the 96-well IMAC purification (SwellGel Nickel Chelated Discs, 96-well filter plate, Pierce, USA) according to the supplier's manual, yielding, on average, more than 1 mg of> 95% pure protein for each clone. Purified proteins were used for all further experiments.

Analytical size-exclusion chromatography (SEC)

All IMAC-purified DARPins were analyzed at a concentration of 10 μM on a Superdex 75 gel-filtration chromatography column (Amersham Pharmacia Biotech, USA) using a Pharmacia SMART system at a flow-rate of 60 μl/min and with PBS as the running buffer (Fig. 2). Phage protein D (gpD) with an apparent mass of 17.6 kDa and phage protein SHP, a trimer with an apparent mass of 50.2 kDa, were used as molecular mass standards.

A subset of DARPins showing too high or too low an elution volume in SEC were further analyzed by SEC multi-angle light-scattering (SEC-MALS) as described.[37]

Competition ELISA measurements

Biotinylated target proteins were bound to neutravidin, which had been immobilized directly onto a MaxiSorp plate as described above. For competition, purified DARPins were incubated with various amounts of non-biotinylated target protein present before (1 h at room temperature) and during the binding reaction (12 min at room temperature). After washing the wells three times with 300 μl of PBSTB, wells were incubated with an anti RGS-His antibody horseradish peroxidase conjugate (QIAgen, Germany) (1:5,000 in PBSTB, 1 h at room temperature) and the bound DARPins were detected with soluble BM Blue POD substrate (Roche Diagnostics, Germany).

Epitope mapping by ELISA

Biotinylated target proteins as well as biotinylated DARPin-pD fusions (see above) were bound to neutravidin, which had been immobilized directly onto a MaxiSorp plate as described above. To the wells with immobilized DARPin-pD fusions the respective non-biotinylated target protein (100 μl, ~20 nM) in PBSTB was added for binding for 1 h at 4 °C. Purified non-biotinylated DARPins (100 μl, ~50 nM) were used for the binding reaction (1 h at room temperature) and detection of bound DARPins was done as described for the ELISA using crude cell extracts (see above).

Surface plasmon resonance (SPR)

SPR was measured by using a BIAcore 3000 instrument (BIAcore, Sweden). All measurements were done in HBST buffer (20 mM HEPES, 150 mM NaCl, 3 mM EDTA pH 7.4, 0.005% Tween-20) at a flow rate of 50 μl/min throughout. The following amounts of biotinylated target protein described in Table 1 were immobilized on SA chips (BIAcore): ErbB1 (1000 RU), ErbB2-509 (400 RU), ErbB4 (600 RU), huIgG1_Fc (500 RU). For the determination of kinetic data, the interactions were measured as follows: 5 min initial buffer flow, followed by a 2 – 15 min injection of DARPin at different concentrations (1 nM – 250 nM) and a final off-rate measurement of 10 to 75 min with buffer flow. The signal of an uncoated reference cell and buffer response was always subtracted from the sensorgrams (double referencing). The kinetic data of the interaction were evaluated with a global fit using Scrubber 2 (BioLogic Software Ltd, USA). (All sensorgrams are shown in Supplementary Data Fig. 2.)

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.07.085

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