nature biotechnology

Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display

Daniel Steiner[1], Patrik Forrer[1,2], Michael T Stumpp[1,2] & Andreas Plückthun[1]

Even proteins that fold well in bacteria are frequently displayed poorly on filamentous phages. Low protein presentation on phage might be caused by premature cytoplasmic folding, leading to inefficient translocation into the periplasm. As translocation is an intermediate step in phage assembly, we tested the display levels of a range of proteins using different translocation pathways by employing different signal sequences. Directing proteins to the cotranslational signal recognition particle (SRP) translocation pathway resulted in much higher display levels than directing them to the conventional post-translational Sec translocation pathway. For example, the display levels of designed ankyrin-repeat proteins (DARPins) were improved up to 700-fold by simply exchanging Sec- for SRP-dependent signal sequences. In model experiments this exchange of signal sequences improved phage display from tenfold enrichment to >1,000-fold enrichment per phage display selection round. We named this method 'SRP phage display' and envision broad applicability, especially when displaying cDNA libraries or very stable and fast-folding proteins from libraries of alternative scaffolds.

Phage display is an *in vitro* selection method that allows polypeptides with desired properties to be extracted from large collections of variants[1,2]. Most commonly, filamentous phages, such as f1, M13 and fd, that infect *Escherichia coli* are used for phage display. Filamentous phages have been intensively investigated for the selection of peptides or antibodies with desired properties from combinatorial libraries[3,4]. Although the major coat protein (protein-8, p8) of the filamentous phage M13, present at ~2,700 copies, and all four minor M13 coat proteins (p3, p6, p7, p9), present at ~5 copies each, have been used for display[2], fusions with p3 have been used most frequently. The peptide or protein of interest (POI) to be displayed is placed between an N-terminal signal sequence and either the whole mature p3 or its C-terminal domain. The POI fused to p3 is translocated into the periplasm by the *E. coli* translocation system while remaining anchored to the cytoplasmic membrane by the C-terminal hydrophobic extension of p3. Upon completion of the extrusion of the phage particle, the p3 fusion protein is incorporated into the tip of the phage coat in a complex with p6 (ref. 5).

Many intra- or extracellular proteins with different sizes and structures have been functionally displayed on filamentous phage[2]. Nevertheless, numerous proteins are only poorly selected from phage display libraries, because they are displayed with very low efficiency or are refractory to display[6]. There are many reasons why a POI might fail to be displayed[6]; for example, (i) the POI can aggregate in the periplasm[7,8] or cytoplasm, sometimes resulting in *E. coli* lysis[9]; (ii) certain disulfide-containing proteins are displayed at lower levels than their nondisulfide-containing counterparts, presumably because of inefficient folding as a result of nonnative disulfide bond formation[10]; (iii) the POI can be refractory to translocation into the periplasm because of incompatibility of local sequence stretches with translocation[11] and (iv) the POI or the linking peptide to p3 can be degraded by periplasmic or cytoplasmic proteases.

Various attempts have been made to circumvent poor display. The host-cell toxicity of the p3 fusion protein can be limited when its expression is reduced[12]. Periplasmic folding of the POI has been improved when phage display vectors coexpressing the periplasmic chaperones Skp[13] or FkpA[14] have been employed. In phagemid/helper phage systems[2], wild-type p3 and the fusion protein compete for incorporation into the phage particles. Therefore, to improve display levels in these phagemid systems, different helper phages have been engineered that encode either no wild-type p3, or defective or proteolysis-sensitive p3 variants[15–18]. Interestingly, the display level of the Stoffel fragment of *Taq* DNA polymerase (*Taq*) was improved by partial randomization of the signal sequence of *Erwinia carotovora* pectate lyase PelB (PelBss) and subsequent selection for increased display, but the mechanism of the improvement was not investigated[18].

Despite these numerous improvements in phage display, many proteins still display poorly[2,6]. DARPins[19,20], which have been successfully selected by ribosome display for specific binding to different target proteins from corresponding combinatorial libraries[21,22], were virtually refractory to display on filamentous phage using a standard phagemid. DARPins have no cysteines, can be expressed in soluble form with high yields in the cytoplasm of *E. coli*, show high thermodynamic stability[20] and fast cooperative folding (S. Wetzel & A.P., unpublished data), and are very resistant to proteolysis (H.K. Binz & A.P., unpublished data). We therefore reasoned that premature cytoplasmic folding of DARPins, and proteins with similar properties,

[1]Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland. [2]Molecular Partners AG, c/o Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland. Correspondence should be addressed to A.P. (plueckthun@bioc.unizh.ch).

Received 30 November 2005; accepted 12 May 2006; published online 2 July 2006; doi:10.1038/nbt1218

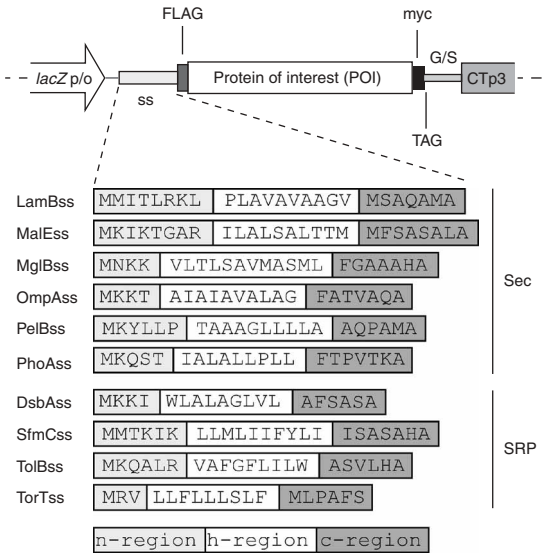

Figure 1 Scheme of the expression cassette of the pDST phagemid vector series. The expression cassette is composed of a promoter/operator element of the *lacZ* gene of *E. coli* (*lacZ* p/o), the coding sequences for the signal sequence (ss) and a protein of interest (POI) to be displayed, a suppressable stop codon (TAG), the coding sequences for a flexible glycine/serine linker (G/S) and for the C-terminal domain (amino acids 250–406) of protein 3 of filamentous phage M13 (CTp3), mediating incorporation of the fusion protein into the phage particle, two stop codons (TGATAA, not depicted) and a transcription terminator element (not depicted). The coding sequence of the POI is flanked by DNA sequences encoding a FLAG-tag (FLAG) and a myc-tag (myc). Shown are the single letter amino acid sequences for the LamB signal sequence (LamBss), the MalE signal sequence (MalEss), the MglB signal sequence (MglBss), the OmpA signal sequence (OmpAss), the PelB signal sequence (PelBss) and the PhoA signal sequence (PhoAss) as representatives of signal sequences targeting the Sec pathway (Sec), and for the DsbA signal sequence (DsbAss), the TorT signal sequence (TorTss), the SfmC signal sequence (SfmCss) and the TolB signal sequence (TolBss) as representatives of signal sequences targeting the SRP pathway (SRP). The positively charged N-terminal region (n-region), the apolar hydrophobic core and the more polar C-terminal region (c-region) of the signal sequences are highlighted. They were assigned by the method described[49], as implemented on the website http://www.cbs.dtu.dk/services/SignalP/. It should be noted that the Sec dependence of PelBss is only putative.

might result in their inefficient translocation into the periplasm and, as a consequence their poor display levels.

Three major pathways[23] are known for the translocation of polypeptides across the cytoplasmic membrane into the periplasm of Gram-negative bacteria: the Sec pathway[23], the signal recognition particle (SRP) pathway[24–26] and the Tat pathway[27,28]. The respective signal sequences strongly favor the targeting of proteins to one of these pathways. The Sec pathway translocates polypeptides post-translationally, whereas the SRP pathway translocates polypeptides cotranslationally. These two pathways converge at the Sec translocon, which transports the polypeptides in an unfolded state across the cytoplasmic membrane. In contrast to these two pathways, the Tat pathway translocates only folded proteins post-translationally through the Tat translocon.

The majority of secreted *E. coli* proteins and phage p3 (ref. 29) use the Sec-dependent translocation pathway. In early phage display experiments using well-secreted proteins, such as antibody fragments, the translocation or display levels showed no major systematic dependence on the signal sequence used, but the only signal sequences tested were Sec dependent[30]. Consequently, bacterial Sec-dependent translocation signal sequences, such as those from PhoA or OmpA, have been used predominantly in phage display.

The Sec pathway can transport only polypeptides that are kept in an unfolded conformation, either by intrinsic features of the protein or by the specialized cytoplasmic chaperone SecB, and perhaps other cytoplasmic chaperones[27]. Therefore, this pathway is inherently incapable of translocating proteins that do not remain in an unfolded state in the cytoplasm. We reasoned that such inefficient translocation could also lead to inefficient display, thus rendering phage display selections very inefficient or even impossible for proteins that fold prematurely in the cytoplasm.

Indeed, the rapidly folding cytoplasmic protein thioredoxin can be translocated into the periplasm only at very low yields using the Sec-dependent PhoA signal sequence (PhoAss)[31]. However, the signal sequence of DsbA (DsbAss) efficiently allowed the translocation of this rapidly folding protein into the periplasm[32]. It has been shown[33] that the reason for this effect is that DsbAss directs the fused protein to the cotranslational SRP pathway, thereby obviating inhibitory effects of cytoplasmic protein-folding on translocation.

We found that DsbAss and other signal sequences that engage the SRP pathway allow the efficient display of DARPins on filamentous phage, and strongly improve the display levels of a range of other proteins. No negative effects of the SRP-directing signal sequences were observed, suggesting that this method, termed SRP phage display, is generally applicable to expand the range of proteins that can be efficiently displayed on filamentous bacteriophage.

RESULTS

Phagemids with Sec- or SRP-type signal sequences

To test whether cotranslational translocation mediated by the SRP pathway would improve display of proteins on filamentous phage particles, compared to the post-translational translocation using the Sec pathway, we constructed two phagemid series, which are identical except for the signal sequence. They contained either the signal sequence of *E. coli* PhoA (PhoAss), directing the p3 fusion proteins to be displayed to the post-translational Sec pathway, or the signal sequence of *E. coli* DsbA (DsbAss)[33], directing the p3 fusion proteins to be displayed to the cotranslational SRP pathway. All further elements of the phagemids and their expression cassettes are described in detail in **Figure 1** and **Supplementary Figure 1** online. To test the applicability of the SRP translocation pathway for phage display, we cloned genes encoding a range of proteins into both phagemids (**Table 1**).

Increased display yields using DsbAss

The display yields of p3 fusion proteins on phage particles were compared between phage particles produced from phagemids differing only in the signal sequence (PhoAss versus DsbAss). Phage particles were purified by CsCl-gradient centrifugation, and the display yields were analyzed by western blot analysis (**Fig. 2a**,**b**). A single-chain Fv antibody (scFv) showed about the same display yield, independent of the signal sequence used. In stark contrast, all four DARPins tested could be efficiently displayed only when using the DsbAss-containing phagemids; when using the PhoAss-containing phagemids, we could not detect displayed protein for the nonbinding DARPin E3_5 (unselected DARPin, without target specificity)[20] or only very low amounts for the other DARPins tested.

To test the scope of this increased display, we compared several other proteins with the DsbAss and PhoAss. The use of

Table 1 Description of proteins tested for display on the surface of filamentous bacteriophage

Protein	Abbr.	Phagemid PhoAss	Phagemid DsbAss	Description[a]	Acc. no.[b], references
scFv_gpD	scFv	pDST24	pDST31	E1 - T245 of single-chain Fv binding to gpD	unpublished
DARPin 3a	3a	pDST22	pDST23	D13 - Q166 of DARPin 3a binding to APH	ref. 22
DARPin JNK2_2_3	2_3	pDST34	pDST37	D13 - Q133 of DARPin JNK2_2_3 binding to JNK2	ref. 21
DARPin E3_5	E3_5	pDST30	pDST32	D13 - Q166 of nonbinding DARPin E3_5	GB: AAO25689, ref. 20
DARPin E3_19	E3_19	pDST65	pDST66	D13 - Q166 of nonbinding DARPin E3_19	GB: AAO25690, ref. 20
GCN4	GCN4	pDST39	pDST40	GCN4 derivative (RMKQLEDKVELLPKNYHLENEVARLKKLVGER)	SP: P03069
pDΔN2	gpD	pDST41	pDST42	T21 - V110 of the capsid stabilizing protein of bacteriophage λ	SP: P03712
JNK2α2	JNK2	pDST45	pDST46	S2 - R424 of c-jun N-terminal kinase 2 (JNK2α2)	SP: P45984
TrxA	TrxA	pDST47	pDST48	S1 - A108 of thioredoxin (*trxA* gene of *E. coli*)	SP: P00274
Taq polymerase	*Taq*	pDST51	pDST52	S290 - E832 Stoffel fragment of *Taq* DNA polymerase	SP: P19821
λ-phosphatase	λPP	pDST53	pDST54	M1 - A221 of bacteriophage λ Ser/Thr protein-phosphatase	SP: P03772
APH	APH	pDST55	pDST56	A2 - F264 of aminoglycoside phosphotransferase (C19S, C156S, S194C)	SP: P0A3Y5

[a]The first and last amino acid used are indicated in single letter amino acid code. [b]Accession numbers: GB, GenBank; SP, Swiss-Prot. DARPin, designed ankyrin-repeat protein; DsbAss, DsbA signal sequence; PhoAss, PhoA signal sequence; scFv, single-chain Fv antibody fragment.

DsbAss-containing phagemids resulted in considerably higher display yields for p3 fusions with the phage λ coat protein D (gpD)[34], thioredoxin (TrxA) and aminoglycoside phosphotransferase IIIa (APH). Slightly higher display yields were observed with the DsbAss for the leucine zipper region of GCN4, *Taq* and the bacteriophage λ protein-phosphatase (λPP). In every case tested to date the display level was either maintained or increased by switching from PhoAss to DsbAss. Displayed c-jun N-terminal kinase 2α2 (JNK2) could not be detected with either signal sequence, suggesting that for this protein, lack of display is not due to premature folding (see below). These results demonstrate that the SRP-dependent DsbAss strongly increased display levels of p3 fusion proteins on phage particles for most polypeptides tested.

To quantify the protein display yields (**Fig. 3**), we analyzed phage particles displaying the target-specific APH-binding DARPin 3a or the JNK2-binding DARPin 2_3 (refs. 21,22) by enzyme-linked immunosorbent assay of phage (phage ELISA) (**Fig. 3a**). Phage particles displaying DARPins specifically binding to the target proteins APH and JNK2 were compared after production under identical conditions from either DsbAss-containing phagemids or PhoAss-containing phagemids. Based on the detection of bound phage particles with an anti-M13 antibody, an increased display yield of up to 600-fold was observed when the SRP-dependent DsbAss was used (**Fig. 3a,d**).

To exclude any dependence on the *E. coli* strain, we confirmed these results of DsbAss causing improved display yields (obtained in *E. coli* XL1-Blue) with another strain. Thus we tested the genotypically different *E. coli* strain TG1, frequently used in phage display (data not shown; for genotypes and strain references, see **Supplementary Table 1** online).

Faster enrichment when using DsbAss

To demonstrate that the higher display yields obtained by using DsbAss also dramatically improved selection experiments, we mixed two test mixtures containing four different types of phage particles produced from phagemids encoding either DsbAss or PhoAss in various ratios. For both test mixtures, phage particles displaying the target-specific DARPins 3a and 2_3 (binding the proteins APH and JNK2, respectively)[21,22] were spiked at a ratio of 1:10^7 into phage

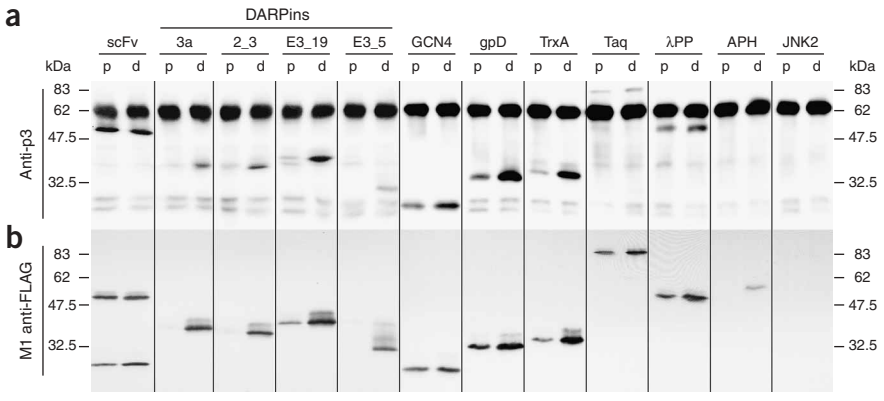

Figure 2 Display yields of the various POIs on phage particles. (**a**,**b**) CsCl-purified phage particles produced by the use of the respective phagemid indicated and normalized by UV absorbance to the same number of phage particles were separated by SDS-PAGE, blotted onto PVDF membranes and detected with antibodies specific for the C-terminal domain of protein 3 (anti-p3) (**a**) or the FLAG-tag (M1 anti-FLAG) located at the N terminus of the POI (**b**). The abbreviated names of the polypeptides are indicated on top of the lanes and refer to the polypeptides listed in **Table 1**. The display yields are compared for each polypeptide using either the PhoA signal sequences (lanes labeled 'p') or the DsbA signal sequence (lanes labeled 'd') translocating the corresponding fusion protein by the Sec pathway or the SRP pathway, respectively. The molecular weights of marker proteins are indicated in kDa at both sides of the blot. The band at 62 kDa in the anti-p3 blot corresponds to wild-type p3 and provides an additional normalization of the amount of phage particles loaded. Wild-type p3 is well known[50] to run at a seemingly higher MW. Note that DARPin E3_5-p3 fusion runs at an apparently lower MW than other DARPin-fusions. This is also observed for this very stable protein in nonfused form, for which mass spectrometry indicated the expected mass, and may indicate incomplete denaturation on the SDS gel, compared to the less stable E3_19 which runs at the expected MW.

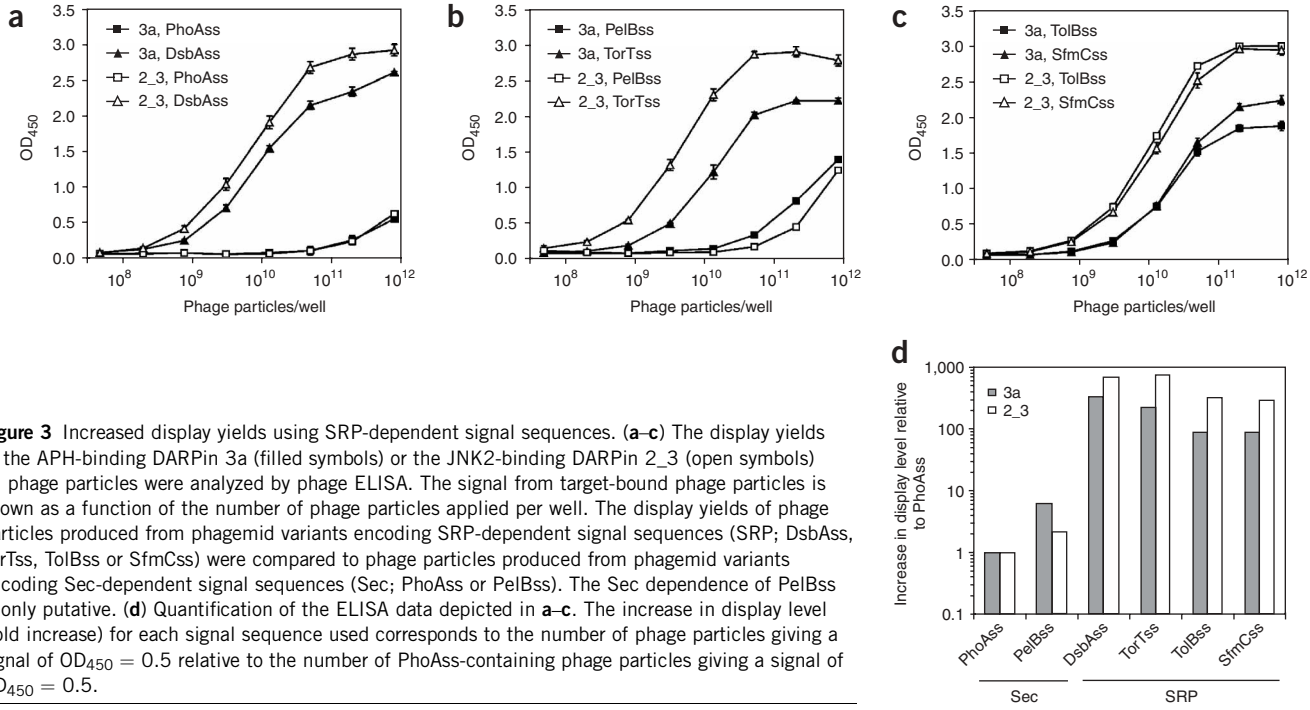

Figure 3 Increased display yields using SRP-dependent signal sequences. (**a**–**c**) The display yields of the APH-binding DARPin 3a (filled symbols) or the JNK2-binding DARPin 2_3 (open symbols) on phage particles were analyzed by phage ELISA. The signal from target-bound phage particles is shown as a function of the number of phage particles applied per well. The display yields of phage particles produced from phagemid variants encoding SRP-dependent signal sequences (SRP; DsbAss, TorTss, TolBss or SfmCss) were compared to phage particles produced from phagemid variants encoding Sec-dependent signal sequences (Sec; PhoAss or PelBss). The Sec dependence of PelBss is only putative. (**d**) Quantification of the ELISA data depicted in **a**–**c**. The increase in display level (fold increase) for each signal sequence used corresponds to the number of phage particles giving a signal of $OD_{450} = 0.5$ relative to the number of PhoAss-containing phage particles giving a signal of $OD_{450} = 0.5$.

particles displaying the nonbinding DARPins E3_5 and E3_19 (unselected DARPins, without target specificity)[20] (**Table 2**). Whereas the APH- and JNK2-specific phage particles could be enriched from the test mixture produced from DsbAss-encoding phagemids by a factor of more than 1,000 per selection cycle (>10% of the tested clones were specific for their target after only two cycles of selection, as determined by PCR with clone-specific primers), the corresponding PhoAss-containing APH- and JNK-specific phagemids were not enriched from the test mixture even after five selection cycles (no specific clones were observed). To quantify the enrichment of phage particles produced from phagemids encoding PhoAss, we spiked the phage particles displaying target-specific DARPins at much lower dilution (1:100 and 1:10) into phage particles displaying the nonbinding DARPins E3_5 and E3_19. One cycle of standard phage display selections on the target proteins APH and JNK2 was performed (**Table 2**). The enrichment of APH- and JNK2-specific phage particles from these test mixtures was less than a factor of 10 per cycle for PhoAss, as calculated from the number of specific clones. This observed improvement in enrichment of >100-fold in DsbAss-containing compared to PhoAss-containing phage particles corresponds well to the several hundredfold increased display level observed by western blot analysis (**Fig. 2**) and quantitative ELISA measurements (**Fig. 3a**,**d**).

Other SRP-type signal sequences increase display

To analyze if the SRP-dependence of the signal sequence is really the key determinant for the observed increased display levels using DsbAss, we tested additional signal sequences known to direct proteins either to the SRP pathway or the Sec pathway. The phagemids encoding the target-specific DARPins 3a and 2_3 (binding APH and JNK2, respectively) were adapted by exchanging the signal sequences. We additionally used the SRP-dependent signal sequences of the *E. coli* proteins TolB (TolBss), SfmC (SfmCss) and TorT (TorTss)[35] (**Fig. 1**). The *E. carotovora* PelB signal sequence (PelBss), which is frequently used in phage display, was also tested and is probably Sec dependent because it lacks a strongly hydrophobic region, a typical feature of SRP-dependent signal sequences[35]. To quantify the difference in display levels, the corresponding phage particles were analyzed by phage ELISA (**Fig. 3b**–**d**) as described above. An increased display yield of up to 700-fold was observed for the SRP-dependent TorTss, compared to the Sec-dependent PhoAss. The SRP-dependent SfmCss and TolBss gave an increased display yield up to 300-fold. The putative Sec-dependent PelBss showed only a two- to sixfold increased display yield, compared to PhoAss. For all SRP-dependent signal sequences the ELISA readings obtained for phages displaying

Table 2 Enrichment of phages displaying target-specific DARPins[a]

			Round of phage selection (positive colonies/number of colonies tested)[b]				
Signal seq. of phagemid	Dilution	Antigen	1st	2nd	3rd	4th	5th
DsbAss	$1:10^7$	APH	0/14	4/16	14/14	–	–
		JNK2	0/14	2/16	14/14	–	–
PhoAss	$1:10^7$	APH	0/11	0/15	0/14	–	0/9
		JNK2	0/14	0/16	0/14	–	0/11
	$1:10^2$	APH	2/16	–	–	–	–
		JNK2	1/16	–	–	–	–
	$1:10^1$	APH	4/16	–	–	–	–
		JNK2	7/16	–	–	–	–

[a]Input mixtures produced from phagemids encoding either PhoAss or DsbAss were produced as described in Methods. To a 1:1 mixture of phage particles displaying the nonbinding DARPins E3_5 and E3_19, phage particles displaying the target-specific DARPins 3a (recognizing APH) or 2_3 (recognizing JNK2) were added in a $1:10^7$ dilution for both mixtures and, additionally, in 1:100 and 1:10 dilutions for the mixtures generated from phagemids encoding the PhoAss. [b]Colonies were screened by PCR using primers specific for the DARPins 3a or 2_3.

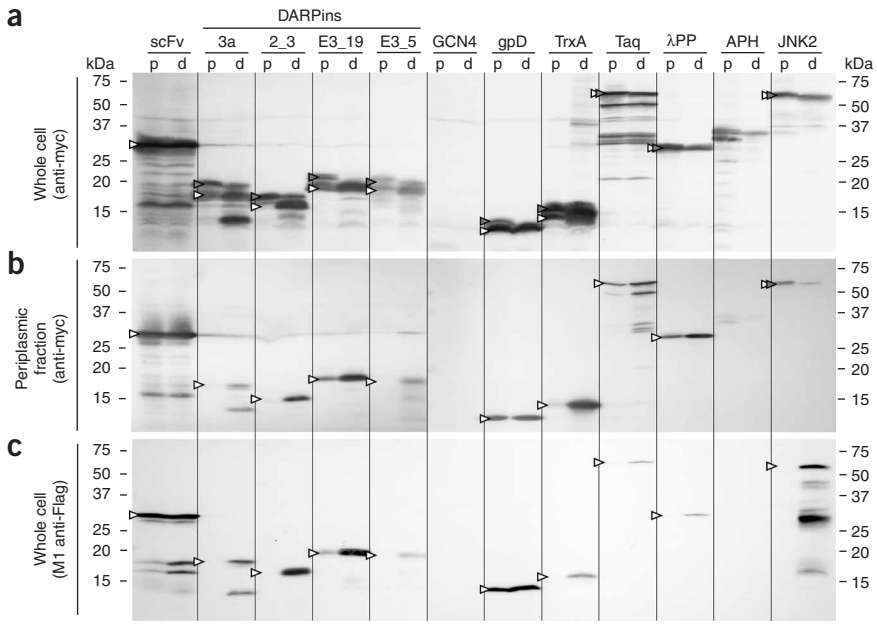

Figure 4 Protein translation and translocation of the various unfused POIs to the periplasm. (**a**–**c**) Whole cell and periplasmic extracts from nonsuppressor *E. coli* JM83 cells, harboring the respective phagemid and normalized to the same amount of cells, were separated by SDS-PAGE, blotted onto PVDF membranes and detected with antibodies specific for the C-terminal myc-tag (anti-myc) (**a**,**b**) and the N-terminal FLAG-tag (M1 anti-FLAG) (**c**). The anti-FLAG antibody M1 selectively recognizes the N-terminal FLAG-tag of the processed, mature POI (**Fig. 1**) having a freely accessible α-amino group. The protein translation and translocation yields are compared for each polypeptide using either the PhoA signal sequence (lanes labeled 'p') or the DsbA signal sequence (lanes labeled 'd') to translocate the corresponding protein by the Sec pathway or the SRP pathway, respectively. The abbreviated names of the polypeptides are indicated on top of the lanes and refer to the polypeptides listed in **Table 1**; for all other abbreviations see **Figure 2**. Mature protein (open triangles) and precursor protein (closed triangles) are indicated where possible. The degradation products of JNK2 detected with the anti-FLAG antibody M1 in whole cell extracts (**c**) could not be detected in the periplasmic fraction with the same antibody (data not shown), suggesting that the degradation products are insoluble and pelleted during centrifugation.

the DARPin 3a were always somewhat lower than for the phages displaying the DARPin 2_3.

Periplasmic levels of POIs correlate with display

We further wanted to investigate whether the increased display of these proteins on phage particles correlates with elevated translocation of these proteins to the periplasm and is not just caused by higher cytoplasmic protein translation yields. We therefore expressed the proteins from identical phagemids as used above (**Table 2**) in the nonsuppressor *E. coli* strain JM83. In contrast to the suppressor strain XL1-Blue, which is used for phage production, this nonsuppressor strain prevents read-through of the amber stop codon after the C terminus of the POI (**Fig. 1**). Whole cell extracts or periplasmic fractions were analyzed by western blotting (**Fig. 4**). To determine the total amount of POI, we used the C-terminal myc-tag of the precursor and mature protein for detection. The amount of periplasmically located, mature POI was additionally determined with the anti-FLAG antibody M1 that selectively recognizes the N-terminal FLAG-tag of the mature POI (**Fig. 1**)—precise cleavage of the signal sequence after translocation is required before this antibody can recognize the freely accessible α-amino group as part of the FLAG tag of the mature POI[36].

Detection of mature and precursor protein with the anti-myc antibody showed that the total protein expression of the different POIs is only influenced to a small extent by the signal sequence (**Fig. 4a**). However, the translocation into the periplasm depends directly on the signal sequence (**Fig. 4b**,**c**). As expected, the signals of the periplasmic fractions detected with the anti-myc antibody (**Fig. 4b**) on western blots correlated well with the signals from whole-cell western blots developed with the anti-FLAG antibody M1 (**Fig. 4c**), which detects only mature protein. The mature protein is thus located in the periplasm. Importantly, precursors of several fusion proteins are accumulating in the cytoplasm when using PhoAss, but not when using DsbAss, as seen in the whole cell blots with detection of the C-terminal myc-tag (**Fig. 4a**).

Independent of the signal sequence used, the scFv antibody fragment is well translocated across the cytoplasmic membrane. In stark contrast, all four tested DARPins could be efficiently translocated only when using the DsbAss-containing phagemids, and almost no protein translocation could be detected when using the PhoAss-containing phagemids. In agreement with previous observations[32,33], the expression of TrxA from DsbAss-containing phagemids resulted in considerably higher translocation yields. Only moderately higher translocation yields were observed for *Taq*, phage λ protein-phosphatase (λPP) and phage λ coat protein D (gpD). The unfused zipper region of GCN4 could not be detected in any of the western blots because of its small size. Very low translocation levels were detected for APH. For JNK2α2, only small amounts of protein were detected in the periplasmic fraction with the anti-myc antibody (JNK2, **Fig. 4b**), whereas a number of insoluble degradation products were detected with the anti-FLAG antibody M1 (JNK2, **Fig. 4c**), suggesting that this degradation may lead to low display (**Fig. 2**).

When these results are compared to those in **Figure 2**, a strong correlation between the translocation yields and the display levels of the different POIs is observed.

Other Sec-type signal sequences cause low display

We analyzed additional signal sequences known to direct proteins to the post-translational Sec-dependent translocation pathway. We chose as the proteins to be displayed DARPin E3_5, a protein with very high thermodynamic stability, and TrxA, a protein previously shown to be efficiently translocated only by SRP-dependent signal sequences[35]. The corresponding phagemids were adapted by exchanging the signal sequences to the SRP-dependent signal sequences (TolBss, SfmCss or TorTss), the Sec-dependent signal sequences (LamBss, MalEss, MglBss or OmpAss)[23] or the putative Sec-dependent PelBss (**Fig. 1**). To compare the difference in display levels, we analyzed the corresponding phage particles by western blotting (**Fig. 5a**). For DARPin E3_5 essentially no displayed protein could be detected with any of the

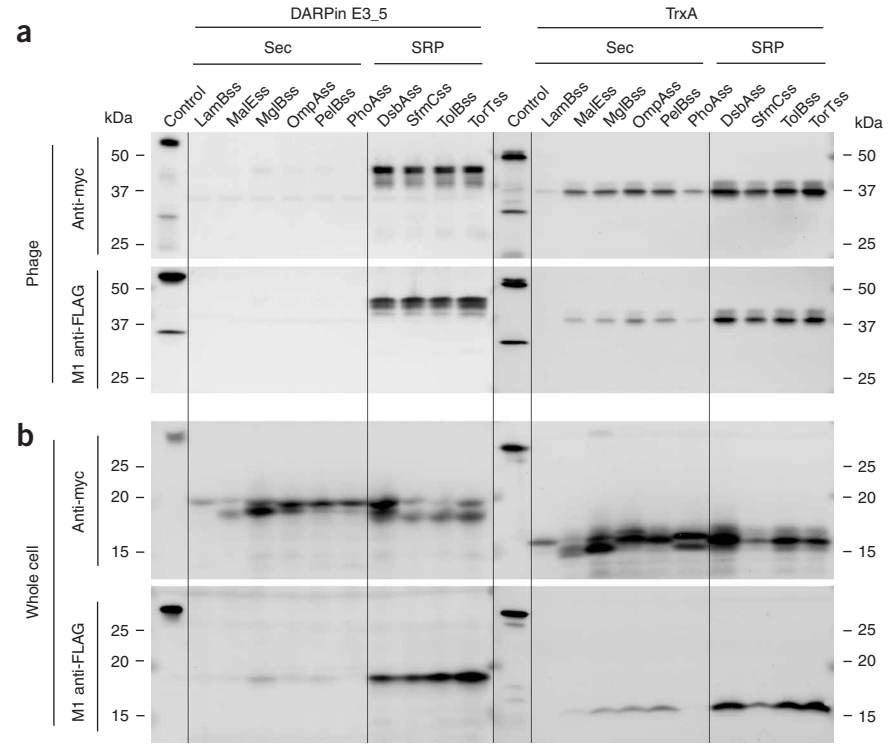

Figure 5 Display yields and periplasmic translocation of DARPin E3_5 and TrxA using various Sec- and SRP-dependent signal sequences. (**a**) Phage particles, produced by the use of the respective phagemid indicated and normalized by UV absorbance to the same number of phage particles, were separated by SDS-PAGE, blotted onto PVDF membranes and detected with antibodies specific for the myc-tag (anti-myc), located at the C terminus of the POI, or the FLAG-tag (M1 anti-FLAG), located at the N terminus of the POI. (**b**) Whole cell extracts from JM83 cells harboring the respective phagemid indicated were analyzed as described for **Fig. 3a**,**c**. The display, protein translation and translocation yields for DARPin E3_5 and TrxA are compared between phagemids encoding Sec-dependent signal sequences (Sec; LamBss, MalEss, MglBss, OmpAss, PelBss or PhoAss) and SRP-dependent signal sequences (SRP; DsbAss, TorTss, TolBss or SfmCss). The Sec dependence of PelBss is only putative. SDS-PAGE of the phage particles produced from phagemids encoding the DARPin E3_5 and whole cell extracts from JM83 cells harboring the phagemids encoding the DARPin E3_5 were run at 60 °C to reduce the partial refolding and therefore smearing of this very stable protein during separation. Control lane: the phagemid encoding the scFv with PhoAss was used as control for western blotting of phage particles or of whole cell extracts.

Sec-dependent signal sequences used, whereas all SRP-dependent signal sequences gave display levels in the same range as found for DsbAss (**Figs. 2** and **5a**). For TrxA the display yields were also considerably higher for all SRP-dependent signal sequences tested than for Sec-dependent signal sequences. However, in contrast to the DARPin E3_5, where almost no displayed protein could be detected for the Sec-dependent signal sequences, TrxA was displayed at moderate levels when using the Sec-dependent MalEss, MglBss and OmpAss and the putative Sec-dependent PelBss, yet substantially lower than with the SRP-dependent signal sequences.

The periplasmic expression yields of the DARPin E3_5 and TrxA correlate well with the display levels observed (**Fig. 5a**,**b**) and are also higher for all SRP-dependent signal sequences than for the Sec-dependent ones. This effect is not caused by increased protein translation (**Fig. 5b**).

DISCUSSION

Some polypeptides are refractory to conventional filamentous phage display because of their individual molecular properties. The reasons have generally not been elucidated[2]. This makes the success of the display of a particular polypeptide unpredictable.

A unique aspect of filamentous phage assembly, in contrast to the assembly of many other bacteriophages, is that it is a secretory process. Incorporation of the coat proteins into the growing phage particle occurs in the cytoplasmic membrane, and nascent phages are extruded from the cell as they assemble[2]. This phage assembly mechanism requires that the POI is translocated across the cytoplasmic membrane into the periplasm to be displayed on the phage particle.

In conventional filamentous phage display, signal sequences directing the POI to the Sec pathway[23] are used for translocation. In this pathway, the polypeptide is first synthesized by the ribosome and then post-translationally translocated, in its unfolded state, by the Sec translocon. This post-translational translocation poses a challenge for keeping the protein in a transport-competent, unfolded state. Transport capability may be achieved by intrinsic features of the polypeptide or by its interaction with the specialized cytoplasmic chaperone SecB and perhaps other cytoplasmic chaperones[27]. However, such mechanisms may not be effective for fast-folding and stable proteins; they may fold prematurely in the cytoplasm making them translocation incompetent.

By merely exchanging the signal sequence targeting the Sec pathway (e.g., LamBss, MalEss, MglBss, OmpAss or PhoAss) by one targeting the SRP pathway (e.g., DsbAss, SfmCss, TolBss and TorTss) in a standard phage display system, we were able to increase the functional display levels for numerous proteins up to 700-fold (**Fig. 3**), resulting in enrichment factors per selection round of more than 1,000 (**Table 2**). This increase of the display levels correlates well with an increase of translocation across the cytoplasmic membrane into the periplasm and is not caused by an overall increase in protein translation (**Fig. 4**).

Similar improvements of periplasmic expression levels when using the SRP pathway instead of the Sec pathway for protein translocation to the periplasm were described with the model protein TrxA[33,35]. Using a total of ten signal sequences, targeting either the Sec or the SRP pathway, we were able to demonstrate in the present study that the improvements in display were indeed a result of the targeted translocation pathway and not caused by individual properties of a particular signal sequence (**Fig. 3**).

For all proteins tested, the display levels on phage particles using SRP-dependent signal sequences were greater than or at least equal to those obtained using the Sec-dependent signal sequences (**Figs. 2** and **5**). This is also true for proteins, such as an scFv-fragment, that are already very well displayed using the conventional Sec-dependent signal sequences. These findings strongly suggest that the use of the cotranslational SRP pathway substantially expands the range of

proteins that can be efficiently displayed on phage particles without having negative effects on already well-displayed proteins.

It is reasonable to assume that cotranslational SRP-dependent translocation obviates the inhibitory effect of premature cytoplasmic protein folding on translocation and thereby allows the display of fast-folding and stable proteins that could not be displayed using the post-translational Sec pathway (**Figs. 2** and **5a**). These latter proteins are efficiently displayed only when SRP-dependent signal sequences are used. Oxidized TrxA, for example, has a high ΔG_o value of 8.1 kcal/mol and a very fast burst phase in folding with a first-order rate constant of 400 s^{-1} (ref. 37).

DARPins possess even higher thermodynamic stabilities than TrxA and have folding rates within the same order of magnitude. Thermal and denaturant-induced unfolding studies revealed T_m values above 66 °C, and ΔG values between 9.5 and 21.1 kcal/mol. In addition, DARPins show fast folding with first-order rate constants around 800 s^{-1} (S.Wetzel & A.P., unpublished data). Interestingly, the less stable DARPin E3_19 (ΔG value of 9.6 kcal/mol) is still displayed at some low level when using Sec-dependent signal sequences, whereas the more stable DARPin E3_5 (ΔG value of 14.8 kcal/mol) is essentially not displayed (**Figs. 2** and **5a**). Interestingly, TrxA variants having slower folding rates and lower thermodynamic stabilities than the wild-type protein also show improved translocation by the Sec pathway[38]. In line with our hypothesis, much higher display levels are seen for TrxA wt when the SRP-dependent signal sequences are used (**Fig. 2**). Whereas the Sec-dependent signal sequences do not enable translocation of DARPin E3_5 and translocate TrxA only at a low level, SRP-dependent signal sequences translocate both proteins, only at a much higher level (**Fig. 5b**).

The SRP-dependent signal sequence only moderately improved the display level for gpD, a protein with a ΔG_o value of 5.1 kcal/mol[39] and a moderate folding rate with the fastest phase estimated at 5 s^{-1} (P.F. & A.P., unpublished data). In contrast, antibody fragments can be translocated and displayed equally well with the Sec- or SRP-dependent signal sequences (**Figs. 2** and **4**). For antibody fragments, no evidence of a decrease in translocation with increasing thermodynamic stability is apparent[40], presumably because the fastest phases of folding of these proteins do not exceed 10 s^{-1} (ref. 41), and folding rates of many such molecules are much smaller.

Proteins that need cytoplasmic cofactors for folding or function may be refractory to display using the SRP translocation pathway. A recently described phage display system based on the post-translational Tat translocation pathway[42] might be better suited for the display of such proteins. Because it has been proposed that p3 itself cannot be displayed with the Tat system[42], the POI may have to be secreted separately and then assembled in the periplasm. A general limitation of this Tat-based approach is that only folded proteins can be translocated through the Tat translocon[43]. For example, disulfide-containing proteins such as antibody fragments can be secreted with the Tat system, and presumably displayed, only from a strain that allows disulfide formation in the cytoplasm[43].

As with any selection technology, the success of phage display selections strongly depends on the diversity of functionally displayed library members. A large combinatorial DNA library alone does not guarantee great functional diversity. We propose that SRP phage display could increase the functional diversity of many libraries, especially those expected to contain members with widely different folding rates and stabilities, such as for example, cDNA libraries. With SRP phage display, selections from such libraries can probably be performed with similar enrichment factors and numbers of selection rounds as typical for 'Sec-compatible' proteins and peptides. The enrichment factor for displaying DARPins was increased from about 10 to over 1,000 by switching to SRP phage display (**Table 2**).

We envision that SRP phage display will be applicable to all variants of phagemid or phage-based systems[12,15–18,44,45], including monovalent or polyvalent display, and will be compatible with all filamentous-phage coat proteins used for display. SRP phage display requires only the POI to be rerouted to the SRP pathway by simply exchanging the signal sequence. CysDisplay[45] and pJuFo phage display[44], two methods in which the POI is disulfide linked but not directly fused to p3, may also benefit from the SRP pathway. In these systems, the POI would be translocated by the SRP pathway, whereas the corresponding phage coat protein could still be translocated by the conventional Sec pathway, in analogy to the strategy described for the Tat pathway[42].

We identified post-translational protein translocation across the cytoplasmic membrane as a major bottleneck of conventional filamentous phage display. By redirecting the fusion proteins to the cotranslational SRP pathway using an appropriate signal sequence, we achieved efficient display of a wide range of POIs, presumably by avoiding premature folding in the cytoplasm. In particular, highly stable and fast-folding POIs were refractory to display using a Sec-dependent signal sequence, but can be efficiently displayed using a SRP-dependent signal sequence. In all cases tested the protein display levels were either maintained or improved by using a SRP-directing signal sequence. Therefore, this method seems to be a simple and broadly applicable way to expand the range of proteins that can be efficiently displayed and selected on filamentous phage.

METHODS

Materials. All chemicals were of the highest quality and purchased from Fluka unless stated otherwise. Oligonucleotides were from Microsynth. Vent DNA polymerase, restriction enzymes and buffers were from New England Biolabs or Fermentas. Helper phage VCS M13 was from Stratagene. All cloning and phage amplification was performed in *E. coli* XL1-Blue from Stratagene. Additionally, *E. coli* TG1 was used for phage production. Periplasmic protein expression was examined in the nonsuppressor *E. coli* strain JM83 obtained from the American Type Culture Collection. Genotypes and references for the *E. coli* strains used are described in the **Supplementary Table 1** online.

Molecular biology. Unless stated otherwise, all molecular biology methods were performed according to standard protocols[46].

Cloning. The phagemid vectors (plasmids with phage origin) that were prepared for the present study are described in detail in the **Supplementary Methods** online. The DNA encoding the proteins to be displayed was inserted into phagemids encoding either PhoAss or DsbAss, resulting in the phagemids listed in **Table 1**. The first and the last amino acids of the cloned proteins of interest are given in **Table 1** as well as the reference or accession number for either GenBank or Swiss-Prot databases.

For the DARPins 3a and 2_3, phagemids encoding the PelBss (pDST80 and pDST81, respectively), SfmCss (pDST86 and pDST87, respectively), TolBss (pDST84 and pDST85, respectively) and TorTss (pDST88 and pDST89, respectively) were generated.

For the DARPin E3_5 and thioredoxin (TrxA), phagemids encoding the LamBss (pDST110 and pDST117, respectively), MalEss (pDST109 and pDST116, respectively), MglBss (pDST111 and pDST118, respectively), OmpAss (pDST103 and pDST104, respectively), PelBss (pDST105 and pDST112, respectively), SfmCss (pDST108 and pDST115, respectively), TolBss (pDST106 and pDST113, respectively) and TorTss (pDST107 and pDST114, respectively) were generated. All signal sequences used are described in detail in **Supplementary Table 2** online.

Phage production and purification. The protocols were adapted from refs. 47 and 48. Five ml 2× YT medium containing 1% glucose, 34 μg/ml

chloramphenicol (cam) and 15 μg/ml tetracycline (tet) was inoculated with a single colony of *E. coli* XL-1 Blue harboring the phagemid of interest, and the cells were grown overnight at 30 °C with shaking. Five ml fresh 2× YT medium containing 1% glucose, 34 μg/ml cam and 15 μg/ml tet was inoculated with the overnight cultures at a ratio of 1:100 ($OD_{600} \approx 0.04$) and grown at 37 °C to an OD_{600} of 0.5 with shaking. The cultures were infected with VCS M13 helper phage at 10^{10} (at plaque forming units per ml (multiplicity of infection ~ 20) and the cells were incubated for 30 min at 37 °C without agitation and then for 30 min at 37 °C with shaking. The medium was changed by harvesting the cells by centrifugation (3,500*g*, 24 °C, 10 min) and resuspending the pellet in 50 ml of 2× YT medium containing 34 μg/ml cam, 50 μg/ml kanamycin (kan) and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). After growth for 14 to 16 h at 30 °C with shaking, the cells were removed by centrifugation (5,600*g*, 4 °C, 10 min).

The culture supernatant was incubated on ice for 1 h with one-fourth volume of ice-cold PEG/NaCl solution (20% polyethyleneglycol (PEG) 6000, 2.5 M NaCl). The precipitated phage particles were then collected by centrifugation (5,600*g*, 4 °C, 15 min) and redissolved in 1 ml of TBS_{150} (25 mM Tris/HCl, 150 mM NaCl, pH 7.5). The phage particles were further purified by CsCl-gradient centrifugation as described[13]. The total concentration of phage particles was quantified spectrophotometrically[48]. The infective titer of the phage samples was determined by titration on *E. coli* XL-1 Blue cells using 2× YT agar plates containing 1% glucose, 34 μg/ml cam and 15 μg/ml tet. The colonies were counted after overnight incubation at 37 °C.

Phage blots. We applied 5×10^{11} phage particles, purified by CsCl gradient, to 15% SDS-PAGE under reducing conditions and transferred them to polyvinylidene fluoride (PVDF) Immobilon-P Transfer Membranes (Millipore) by electroblotting. The membranes were blocked with $MTTBS_{150}$ (TBS_{150}, 0.1% Tween-20, 5% skimmed milk) for 1 h at about 22 °C and incubated with a mouse anti-p3 antibody (MoBiTec) (1:1,000 in $MTTBS_{150}$, 20 min at about 22 °C) as primary antibody, which recognizes the C-terminal domain of p3. A $F(ab')_2$ fragment goat anti-mouse IgG horseradish peroxidase conjugate (Pierce) (1:10,000 in $MTTBS_{150}$, 1 h at about 22 °C) was used as the secondary antibody. The proteins were detected with ChemiGlow West substrate (Alpha Innotech).

In a second experiment, the blocked membranes were incubated with mouse anti-FLAG antibody M1 (Sigma) (1:5,000 in $MTTBS_{150}$, 1 h at about 22 °C) as primary antibody. A goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) (1:10,000 in $MTTBS_{150}$, 1 h at about 22 °C) was used as secondary antibody. The proteins were detected with the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Phage ELISAs. Phage ELISAs were carried out to assay the amount of functionally displayed DARPins on M13 phage particles. The targets, biotinylated APH and JNK2 proteins[21], were immobilized as follows: Neutravidin (66 nM, 100 μl/well; Pierce) in TBS_{150} was immobilized on MaxiSorp plates (Nunc) by overnight incubation at 4 °C. The wells were blocked with 300 μl $BTTBS_{150}$ (TBS_{150}, 0.1% Tween-20, 1% BSA) for 1 h at about 22 °C. Binding of the biotinylated APH and JNK2 proteins (100 μl, ≈ 1 μM) in $BTTBS_{150}$ was done for 1 h at 4 °C.

Dilution series of phage particles in $BTTBS_{150}$ were pipetted to the wells and incubated at about 22 °C for 2 h. After washing the wells five times with 300 μl $TTBS_{150}$ (TBS_{150}, 0.1% Tween-20) for 5 min, bound phage particles were detected with mouse anti-M13 antibody horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and soluble BM Blue POD substrate (Roche Diagnostics).

Phage panning. Phage particles displaying DARPins E3_5, E3_19, 3a and 2_3 were produced from either phagemids encoding the PhoAss (pDST30, pDST65, pDST22 and pDST34, respectively) or from phagemids encoding the DsbAss (pDST32, pDST66, pDST23 and pDST37, respectively). These phage particles were subsequently mixed according to the experiment. To a 1:1 mixture of phage particles displaying the nonbinding DARPins E3_5 and E3_19, phage particles displaying the target-specific DARPins 3a or 2_3 were added in $1:10^7$ dilutions (for phage particles produced from phagemids encoding the PhoAss, additionally in 1:100 and 1:10 dilutions).

Biotinylated APH and JNK2 proteins were coated as described for the phage ELISAs. To each well, 0.1 ml of phage particle mixtures (10^{13} colony-forming units/ml) and 0.1 ml $BTTBS_{150}$ were added and incubated for 2 h. After washing with $TTBS_{150}$ (3× for the first selection cycle, 4× for the second cycle and 5× for additional cycles) for 5 min and with TBS_{150} (3× for the first selection cycle, 4× for the second cycle and 5× for additional cycles) for 5 min, the phage particles were eluted by incubating for 15 min with 0.2 ml elution buffer (0.2 M glycine/HCl, pH 2.2) at about 22 °C, followed by an elution for 30 min with 0.2 ml trypsin (10 mg/ml in TBS_{150}) at 37 °C. The combined eluates (neutralized with 10 μl of 2 M Tris-base) were used for the infection of 4 ml of exponentially growing *E. coli* XL1-Blue cells. After 30 min at 37 °C without agitation and 30 min at 37 °C with shaking, the cells were spread on 2× YT agar plates containing 1% glucose and 34 μg/ml cam and 15 μg/ml tet and grown overnight at 37 °C. The cells were washed from the plates with 2× YT containing 1% glucose, 15% glycerol, 34 μg/ml cam and 15 μg/ml tet and used for the phage production for the next cycle of panning. After each panning cycle, the identity of 9 to 16 eluted phage particles was determined. This was done by infection of *E. coli* with these phage particles and screening of the colonies by PCR with clone-specific primers.

Western blot analysis of cellular extracts. Five ml 2× YT medium containing 1% glucose, 34 μg/ml cam and 15 μg/ml tet were inoculated with a single colony of *E. coli* JM83 harboring the phagemid of interest and grown overnight at 30 °C with shaking. Fresh 5 ml 2× YT medium containing 1% glucose, 34 μg/ml cam and 15 μg/ml tet were inoculated with the overnight cultures at a ratio of 1:100 ($OD_{600} \approx 0.04$) and grown at 37 °C to an OD_{600} of 0.5. The medium was changed by harvesting the cells by centrifugation (3,500*g*, 24 °C, 10 min) and resuspending the pellet in 50 ml of 2× YT medium containing 34 μg/ml cam and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). After growth for 14 to 16 h at 30 °C with shaking, the cells were harvested by centrifugation (5,600*g*, 4 °C, 10 min) for the whole cell extracts or treated as described[35] for subcellular fractionation.

Western blot analysis of the whole cell extracts and periplasmic fractions was carried out as described above. The blocked membrane was either incubated with mouse anti-FLAG antibody M1 (Sigma) (1:5,000 in $MTTBS_{150}$, 1 h at about 22 °C) or mouse anti-myc antibody (Cell Signaling Technology) (1:1,000 in $MTTBS_{150}$, 1 h at about 22 °C) as primary antibodies, then goat anti-mouse IgG alkaline phosphatase conjugate as secondary antibody. The proteins were detected with the substrates BCIP and NBT.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

We thank Patrick Amstutz and H. Kaspar Binz for valuable discussions and J. Beckwith and D. Huber for advice on experimental procedures. This work was supported by the Swiss National Center of Competence in Research (NCCR) in Structural Biology and by KTI Discovery.

AUTHOR CONTRIBUTIONS

A.P. conceived the project; D.S., P.F., M.T.S. and A.P. designed experiments; D.S. performed the experiments; D.S., P.F., M.T.S. and A.P. analyzed the data; D.S., P.F., M.T.S. and A.P. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

Published online at http://www.nature.com/naturebiotechnology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Smith, G.P. & Petrenko, V.A. Phage Display. *Chem. Rev.* **97**, 391–410 (1997).
2. Russel, M., Lowman, H.B. & Clackson, T. Introduction to phage biology and phage display. in *Phage Display: A Practical Approach* (eds. Lowman, H.B. & Clackson, T.) 1–26, (Oxford University Press, New York, USA, 2004).
3. Sidhu, S.S., Lowman, H.B., Cunningham, B.C. & Wells, J.A. Phage display for selection of novel binding peptides. *Methods Enzymol.* **328**, 333–363 (2000).
4. Bradbury, A.R. & Marks, J.D. Antibodies from phage antibody libraries. *J. Immunol. Methods* **290**, 29–49 (2004).
5. Rakonjac, J., Feng, J. & Model, P. Filamentous phage are released from the bacterial membrane by a two-step mechanism involving a short C-terminal fragment of pIII. *J. Mol. Biol.* **289**, 1253–1265 (1999).

6. Wilson, D.R. & Finlay, B.B. Phage display: applications, innovations, and issues in phage and host biology. *Can. J. Microbiol.* **44**, 313–329 (1998).
7. Deng, S.J. *et al.* Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *J. Biol. Chem.* **269**, 9533–9538 (1994).
8. Jung, S. & Plückthun, A. Improving *in vivo* folding and stability of a single-chain Fv antibody fragment by loop grafting. *Protein Eng.* **10**, 959–966 (1997).
9. Krebber, A., Burmester, J. & Plückthun, A. Inclusion of an upstream transcriptional terminator in phage display vectors abolishes background expression of toxic fusions with coat protein g3p. *Gene* **178**, 71–74 (1996).
10. Brinkmann, U., Chowdhury, P.S., Roscoe, D.M. & Pastan, I. Phage display of disulfide-stabilized Fv fragments. *J. Immunol. Methods* **182**, 41–50 (1995).
11. Rodi, D.J., Soares, A.S. & Makowski, L. Quantitative assessment of peptide sequence diversity in M13 combinatorial peptide phage display libraries. *J. Mol. Biol.* **322**, 1039–1052 (2002).
12. Krebber, A. *et al.* Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J. Immunol. Methods* **201**, 35–55 (1997).
13. Bothmann, H. & Plückthun, A. Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat. Biotechnol.* **16**, 376–380 (1998).
14. Bothmann, H. & Plückthun, A. The periplasmic *Escherichia coli* peptidylprolyl cis,-trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. *J. Biol. Chem.* **275**, 17100–17105 (2000).
15. Kramer, R.A. *et al.* A novel helper phage that improves phage display selection efficiency by preventing the amplification of phages without recombinant protein. *Nucleic Acids Res.* **31**, e59 (2003).
16. Baek, H., Suk, K.H., Kim, Y.H. & Cha, S. An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res.* **30**, e18 (2002).
17. Rondot, S., Koch, J., Breitling, F. & Dübel, S. A helper phage to improve single-chain antibody presentation in phage display. *Nat. Biotechnol.* **19**, 75–78 (2001).
18. Jestin, J.L., Volioti, G. & Winter, G. Improving the display of proteins on filamentous phage. *Res. Microbiol.* **152**, 187–191 (2001).
19. Forrer, P., Stumpp, M.T., Binz, H.K. & Plückthun, A. A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett.* **539**, 2–6 (2003).
20. Binz, H.K., Stumpp, M.T., Forrer, P., Amstutz, P. & Plückthun, A. Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol.* **332**, 489–503 (2003).
21. Binz, H.K. *et al.* High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat. Biotechnol.* **22**, 575–582 (2004).
22. Amstutz, P. *et al.* Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J. Biol. Chem.* **280**, 24715–24722 (2005).
23. Fekkes, P. & Driessen, A.J. Protein targeting to the bacterial cytoplasmic membrane. *Microbiol. Mol. Biol. Rev.* **63**, 161–173 (1999).
24. Koch, H.G., Moser, M. & Müller, M. Signal recognition particle-dependent protein targeting, universal to all kingdoms of life. *Rev. Physiol. Biochem. Pharmacol.* **146**, 55–94 (2003).
25. Valent, Q.A. Signal recognition particle mediated protein targeting in *Escherichia coli*. *Antonie Van Leeuwenhoek* **79**, 17–31 (2001).
26. Luirink, J. & Sinning, I. SRP-mediated protein targeting: structure and function revisited. *Biochim. Biophys. Acta* **1694**, 17–35 (2004).
27. Fisher, A.C. & DeLisa, M.P. A little help from my friends: quality control of presecretory proteins in bacteria. *J. Bacteriol.* **186**, 7467–7473 (2004).
28. Robinson, C. & Bolhuis, A. Tat-dependent protein targeting in prokaryotes and chloroplasts. *Biochim. Biophys. Acta* **1694**, 135–147 (2004).
29. Rapoza, M.P. & Webster, R.E. The filamentous bacteriophage assembly proteins require the bacterial SecA protein for correct localization to the membrane. *J. Bacteriol.* **175**, 1856–1859 (1993).
30. Plückthun, A. *et al.* in *Antibody Engineering,* edn. 1 (eds. McCafferty, J., Hoogenboom, H.R. & Chiswell, D.J.) 203–252, (IRL Press, Oxford, 1996).
31. Debarbieux, L. & Beckwith, J. The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the *Escherichia coli* periplasm. *Proc. Natl. Acad. Sci. USA* **95**, 10751–10756 (1998).
32. Jonda, S., Huber-Wunderlich, M., Glockshuber, R. & Mössner, E. Complementation of DsbA deficiency with secreted thioredoxin variants reveals the crucial role of an efficient dithiol oxidant for catalyzed protein folding in the bacterial periplasm. *EMBO J.* **18**, 3271–3281 (1999).
33. Schierle, C.F. *et al.* The DsbA signal sequence directs efficient, cotranslational export of passenger proteins to the *Escherichia coli* periplasm via the signal recognition particle pathway. *J. Bacteriol.* **185**, 5706–5713 (2003).
34. Yang, F. *et al.* Novel fold and capsid-binding properties of the lambda-phage display platform protein gpD. *Nat. Struct. Biol.* **7**, 230–237 (2000).
35. Huber, D. *et al.* Use of thioredoxin as a reporter to identify a subset of *Escherichia coli* signal sequences that promote signal recognition particle-dependent translocation. *J. Bacteriol.* **187**, 2983–2991 (2005).
36. Slootstra, J.W., Kuperus, D., Plückthun, A. & Meloen, R.H. Identification of new tag sequences with differential and selective recognition properties for the anti-FLAG monoclonal antibodies M1, M2 and M5. *Mol. Divers.* **2**, 156–164 (1997).
37. Georgescu, R.E., Li, J.H., Goldberg, M.E., Tasayco, M.L. & Chaffotte, A.F. Proline isomerization-independent accumulation of an early intermediate and heterogeneity of the folding pathways of a mixed alpha/beta protein, *Escherichia coli* thioredoxin. *Biochemistry* **37**, 10286–10297 (1998).
38. Huber, D. *et al.* A selection for mutants that interfere with folding of *Escherichia coli* thioredoxin-1 in vivo. *Proc. Natl. Acad. Sci. USA* **102**, 18872–18877 (2005).
39. Forrer, P., Chang, C., Ott, D., Wlodawer, A. & Plückthun, A. Kinetic stability and crystal structure of the viral capsid protein SHP. *J. Mol. Biol.* **344**, 179–193 (2004).
40. Ewert, S., Huber, T., Honegger, A. & Plückthun, A. Biophysical properties of human antibody variable domains. *J. Mol. Biol.* **325**, 531–553 (2003).
41. Jäger, M., Gehrig, P. & Plückthun, A. The scFv fragment of the antibody hu4D5–8: evidence for early premature domain interaction in refolding. *J. Mol. Biol.* **305**, 1111–1129 (2001).
42. Paschke, M. & Höhne, W. A twin-arginine translocation (Tat)-mediated phage display system. *Gene* **350**, 79–88 (2005).
43. DeLisa, M.P., Tullman, D. & Georgiou, G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *Proc. Natl. Acad. Sci. USA* **100**, 6115–6120 (2003).
44. Crameri, R., Hemmann, S. & Blaser, K. PJuFo: a phagemid for display of cDNA libraries on phage surface suitable for selective isolation of clones expressing allergens. *Adv. Exp. Med. Biol.* **409**, 103–110 (1996).
45. Löhning, C., Urban, M. & Knappik, A. Novel methods for displaying (poly) peptides/proteins on bacteriophage particles via disulfide bonds Patent WO0105950 (2001).
46. Sambrook, J. & Russell David, W. (eds.). *Molecular Cloning: A Laboratory Manual,* edn. 3. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001).
47. Clackson, T. & Lowman, H.B.. *Phage Display: A Practical Approach* (Oxford University Press, New York, 2004).
48. Barbas, C.F., III, Burton, D.R., Scott, J.K. & Silvermann, G.J.. *Phage Display: A Laboratory Manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 2001).
49. Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1–6 (1997).
50. Gailus, V. & Rasched, I. The adsorption protein of bacteriophage fd and its neighbour minor coat protein build a structural entity. *Eur. J. Biochem.* **222**, 927–931 (1994).