Selection for a periplasmic factor improving phage display and functional periplasmic expression

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The efficiency of both phage display in *Escherichia coli* and periplasmic expression of recombinant proteins may be limited by the same periplasmic folding steps. To search for *E. coli* factors that improve the efficiency of both procedures, a library of *E. coli* proteins was coexpressed in a phagemid vector that contained a poorly folding single-chain Fv antibody (scFv) fragment fused to g3p. We enriched, by panning for antigen binding, those phagemids in which the amount of displayed scFv is highest. We thus identified the periplasmic protein Skp/OmpH/HlpA as improving phage display of a wide range of scFv fragments. This occurs as a result of an increase in the amount of hybrid protein displayed on the phage. Coexpression of *skp* also increases the functional yield of scFv fragments when expressed by secretion to the periplasm.

Keywords: protein expression, single-chain antibody, *Escherichia coli*

Expression in the bacterial periplasm is the most convenient route to express foreign recombinant proteins containing disulfides, since the bacterial disulfide forming and isomerization machinery[1] can be used. Nevertheless, not all proteins can be produced with high functional yield in the *Escherichia coli* periplasm, and consequently, there has been great interest in the question of the existence of periplasmic chaperones. Unlike the well-characterized cytoplasmic machinery of *E. coli*, DnaK/DnaJ/GrpE, and GroEL/GroES and possibly others[2-4], the chaperone composition of the periplasm is poorly understood[5,6]. While progress in elucidating the signal transduction of periplasmic stress has been made[7-10], the ultimate effector molecules controlling periplasmic folding have remained obscure.

Phage display has been used to screen libraries of peptides as well as a large variety of proteins[11,12]. The displayed proteins are fused to the N-terminus of the whole gene-3-protein (g3p) or that of its C-terminal domain. These fusion proteins fold in the periplasm, while remaining anchored to the inner membrane by the C-terminal hydrophobic extension of g3p, before being incorporated into the phage coat. Therefore, the g3p fusion-proteins will almost certainly fold in the same environment and use the same machinery as periplasmically expressed proteins.

We used this hypothesis to model a search for cellular factors that might aid both the folding of periplasmic proteins and proteins displayed on phage. We used a very poorly folding single-chain Fv fragment (scFv) of the antibody 4-4-20 (refs. 13,14), specific for fluorescein, as a model. This scFv strongly aggregates in the bacterial periplasm, even though the same protein in the native state is very soluble and stable[15]. The folding yield is determined by kinetics rather than thermodynamics, and can thus potentially be helped by cellular factors.

We wished to identify such factors without regard as to whether they are membrane-bound, periplasmic, or even cytoplasmic proteins. We also wanted to find any factors that might affect the total yield of the product without directly influencing folding. We therefore developed a selection system making use of phage display (Fig. 1A). The poorly folding scFv fragment was displayed as a fusion protein with g3p, and a library of *E. coli* proteins was coexpressed on the same phagemid. If a particular *E. coli* cell produces a beneficial factor encoded on this phagemid, this cell will give rise to phage with a selective advantage, because a higher fraction of the phage will display correctly folded scFv. Thus, while the displayed scFv molecule is genetically identical on all phage, this method selects for the effect of the additional factor encoded on the same phagemid, even though the factor itself is not displayed. This factor improves the quality of the scFv, namely the percentage of correctly folded molecules displayed on the phage, by acting on the host cell that produces that particular phage.

Using this strategy (Fig. 1), only a single gene from *E. coli* was enriched, coding for the periplasmic protein Skp, which had been suspected to have a role in folding or transport of outer-membrane proteins[16].

Results

Phage selection and identification of coexpressed factor. We used a phagemid displaying the poorly folding scFv fragment of the antifluorescein antibody 4-4-20 (refs. 13,15) as the recipient for an *E. coli* genomic library. *E. coli* DNA was size-fractionated from 1 to 6 kb and ligated into a polylinker of a phagemid developed for phage display[17]. Thus, *E. coli* genes, regulated under their own promoters, were overexpressed on the phagemid, primarily through an effect of vector copy number. A library size of 5×10^4 clones ensured that each piece of the *E. coli* genome would be represented, provided it led to viable clones.

Seven panning rounds on BSA-fluorescein were carried out (Fig. 1), and after each round the phagemid DNA was cut with the restriction enzyme NotI to detect the accumulation of any inserts. A band of about 990 bp accumulates throughout the panning (Fig. 2). Four of eight single colonies analyzed after the seventh round carried this insert, which was sequenced and identified to contain the gene for the periplasmic protein Skp[18], from 272 bases upstream of the start codon to 199 bases downstream of the stop codon (Fig.

3). Four bases after the stop codon of *skp* is the start codon of *lpxD* (*firA*), leading to a truncated peptide of the 65 N-terminal amino acids (aa) of this protein. One hundred twenty-five base pairs upstream of the start codon of the *skp* gene lies the stop codon of an open reading frame that codes for an 810 aa protein with unknown function. A homology search showed 66.2% similarity to the protective surface antigen D15 of *Haemophilus influenzae* (Swiss-Prot: P46024), and homology to surface proteins of *Pasteurella multocida* (TREMBL: Q51930), *Neisseria gonorrhoeae* (TREMBL: P95359), and *Brucella abortus* (TREMBL: Q44629)

Characterization of the influence of Skp on phage display. To determine how and why Skp gets enriched, we first characterized the phages produced in the absence and presence of coexpressed *skp*. For this purpose, we cloned a variety of different scFv fragments in the phagemid with and without *skp*. The phage titer was indistinguishable within experimental error (data not shown), demonstrating that *skp* was not selected because it would lead to the production of more phages. The antigen-binding phage enzyme-linked immunosorbent assay (ELISA) signal from the same number of purified phage particles was higher, proving that *skp* overexpression increased the number of functional antibody molecules on the phage (Fig. 4). This effect was seen with all four antibodies tested, albeit to different degrees. We also showed that the biological effect is due to the *skp* gene itself by PCR-amplifying the gene without the *IpxD* fragment and recloning it at the same position (data not shown).

We then determined whether the total amount of fusion protein per phage is also increased by the overexpression of *skp*. For this purpose we analyzed the amount of full-length fusion protein on purified phage particles in the presence and absence of *skp* on the phagemid by Western blot, using the monoclonal antibody 10C3 (ref. 19) (Fig. 5). For the scFv 4-4-20, the coexpression of *skp* dramatically increased the presence of fusion protein presented on the phage. As the same amount of purified phage was loaded on the gel, Skp must have facilitated the incorporation of functional fusion protein into the phage, which was also reflected by the antigen-binding ELISA (see above). Skp had this effect on all of the six antibodies tested, albeit to a different extent and to a different final level of incorporation. Because the fusion protein is still only a minor species when compared with wild-type (g3p wt) (encoded by the helper phage), we could not reliably determine a decrease of g3p wt, but most likely, the scFv-g3p fusion protein takes the place of g3p wt more often in the presence of overexpressed Skp.

Characterization of the influence of Skp on soluble periplasmic expression. We examined the effect of Skp on the production of several of the scFv fragments in soluble form using the nonsuppressor strain JM83. The amount of soluble scFv was dramatically increased in the presence of coexpressed Skp as determined by antigen-binding ELISA (Fig. 6). Very similar results were obtained when, instead of the original insert shown in Figure 3, the *skp* gene was PCR-cloned without the *IpxD* fragment (data not shown). To demonstrate that the effect of Skp is also reflected in the yield of purified protein, the scFv fragment of the antiphosphorylcholine binding antibody McPC603-H11 (ref. 20) was tested. Coexpression of *skp* increased the amount of protein purified by affinity-chromatography on phosphorylcholine by about a factor of four.

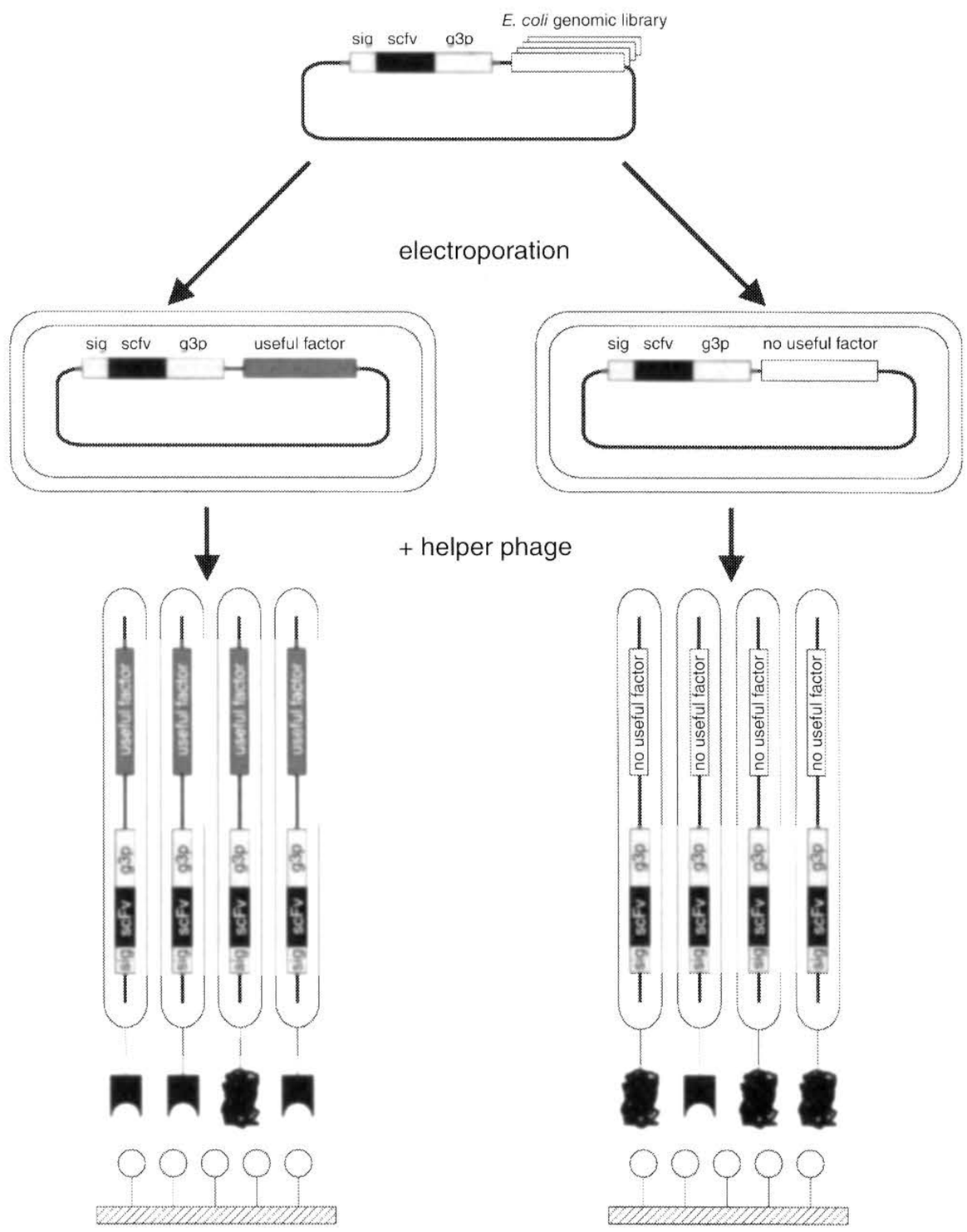

Figure 1. Selection scheme. Principle of selection. An *E. coli* genomic library is coexpressed with an scFv-fragment, fused to g3p. While the antibody is the same throughout, its folding yield varies depending on the coexpressed factor. This factor is not displayed on the phage, but expressed in the host cell producing the phage, which in the case of an useful factor leads to better "quality" scFv fragments displayed. Because the gene for the factor is encoded on the phage, it becomes enriched by phage panning on antigen.

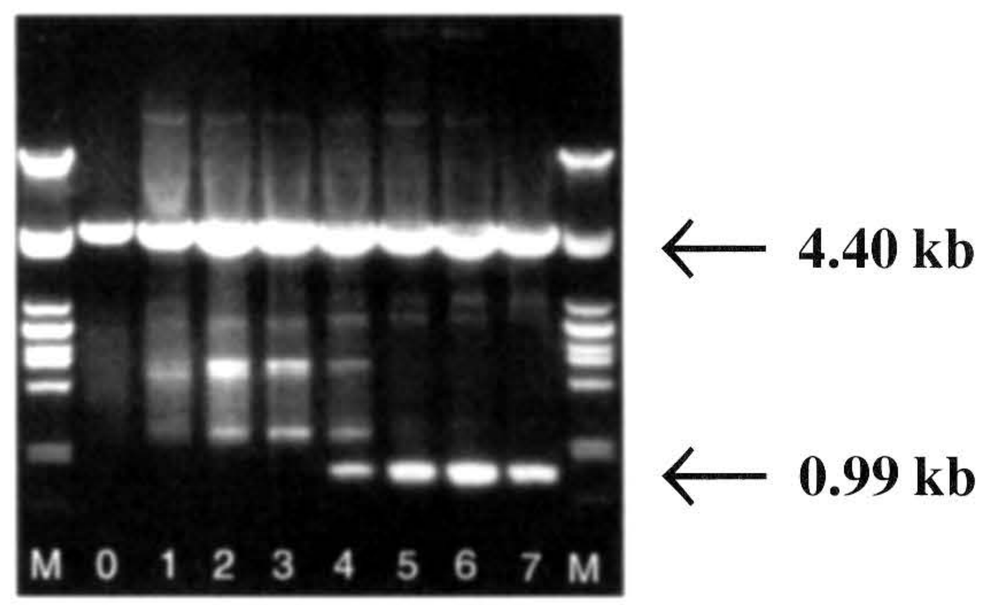

Figure 2. Analysis of phagemid pools after different panning rounds. The phagemid pools were analyzed by restriction enzyme digest with NotI. M: PstI digested λ-DNA as molecular weight marker; lanes 1–7: panning round from which phagemid were isolated; lane 0: phagemids before the first panning round.

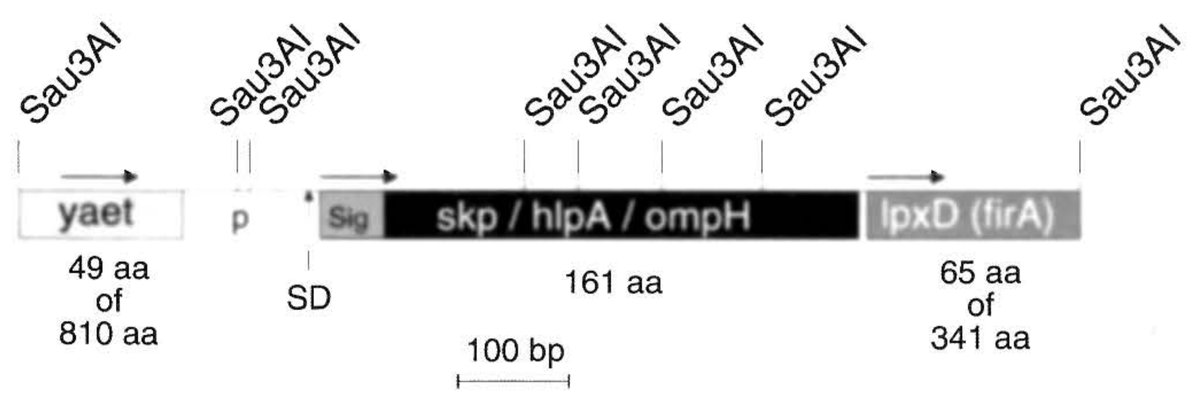

Figure 3. Schematic representation of the 952-bp insert enriched by phage display and panning. Yaet is the product of an open reading frame (ORF) of 810 amino acids of unknown function. *IpxD* codes for UDP-3-O-[3-hydroxymyristoyl]-glucosamine-N-acyltransferase. The only complete ORF found on this insert is the gene *skp*. *SD*: Shine-Dalgarno sequence; *p:* promoter region predicted by neural network analysis[45]. A putative binding site for the factor CpxR, involved in periplasmic stress response, is predicted around the stop codon of *yeat*[7].

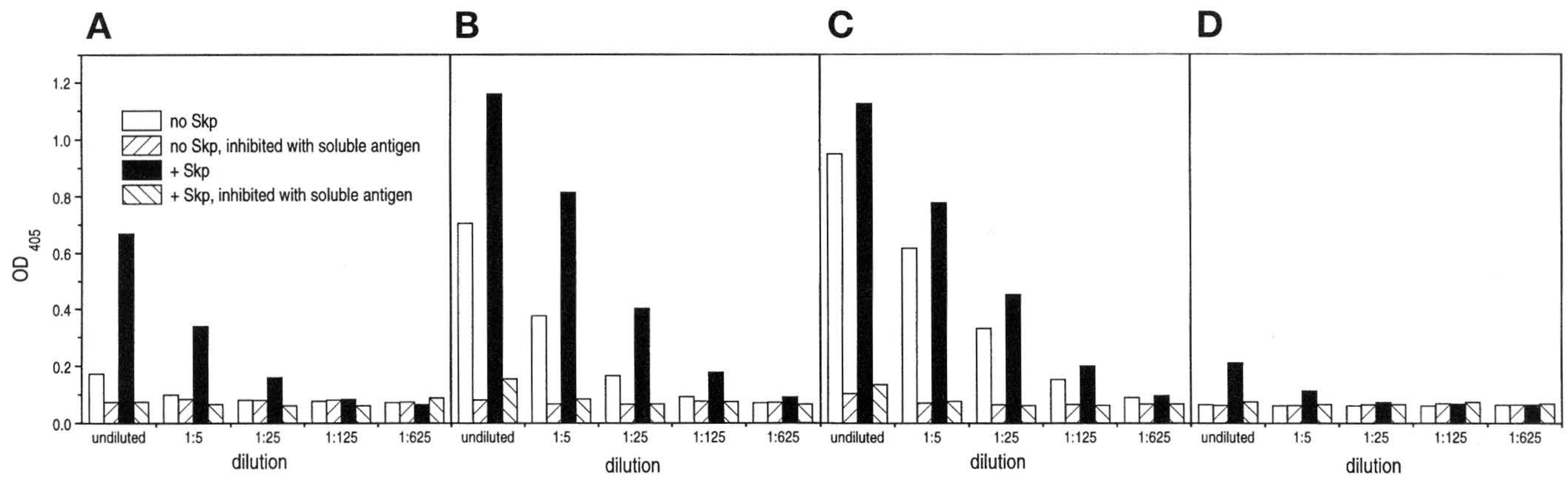

Figure 4. Antigen-binding ELISAs of phage grown with or without overexpressed Skp, displaying the scFv fragments (A) 4-4-20 (ref. 15), (B) 4D5Flu (ref. 25), (C) FITC-E2 (refs. 21 and 22) and ABPC48-C(H22)S (refs. 23 and 24). Phage were purified by CsCl gradients.

The more an scFv fragment tends to aggregate in the periplasm of *E. coli*, the stronger the influence of Skp in the phage ELISA (Figs. 4 and 5). For the scFv fragment of FITC-E2 (refs. 21,22), which shows little insoluble material when expressed in the periplasm, we observed almost no influence of Skp in the phage ELISA, compared with the poorly folding scFv 4-4-20 (ref. 15) and ABPC48-C(H22)S (refs. 23,24). The engineered Flu4D5 (ref. 25), with improved properties compared with 4-4-20, is intermediate. The better an scFv is functionally expressed and the less aggregation-prone it is, the less is the influence of Skp, suggesting that Skp supports the correct folding of poorly expressed scFv fragments and its fusion proteins.

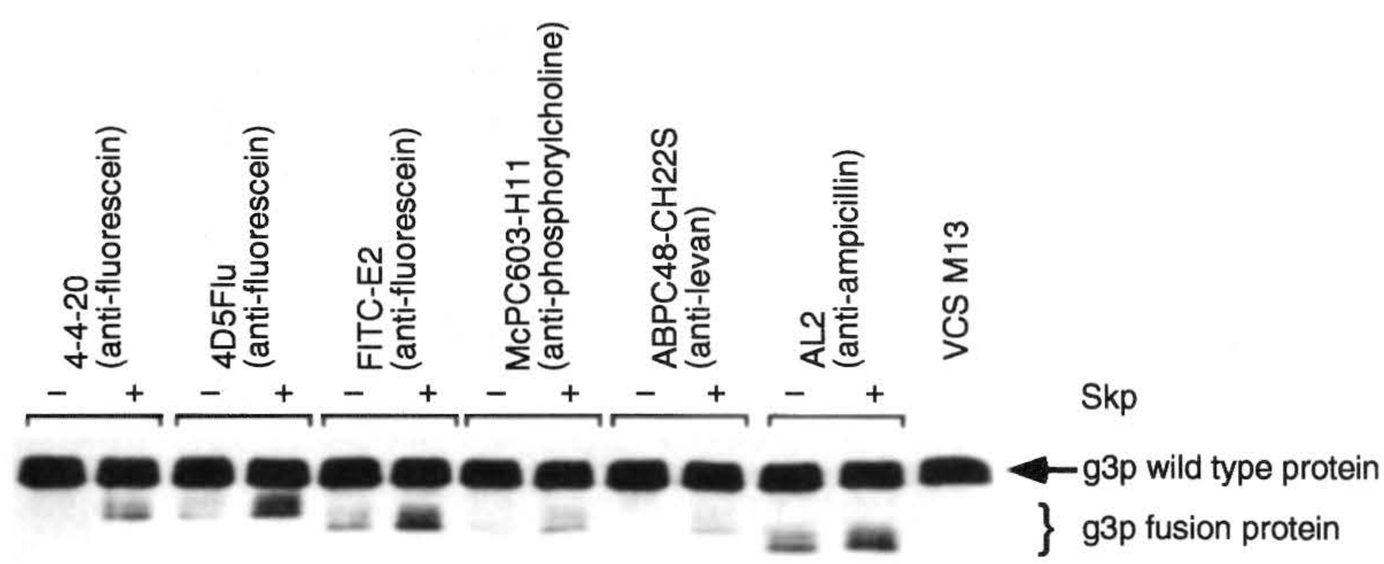

Figure 5. Phage blot. Phage carrying g3p-fusion of the scFv fragments of indicated antibodies were grown with or without overexpressed *skp*. Phage were purified by CsCl gradients. Helper phage VCS M13 was used as size reference for wild-type g3p. The scFv is fused to the C-terminal domain of g3p, and thus the fusion protein runs below wt g3p on SDS-PAGE[19]. The different scFv phages are described in refs. 15, 17, and 21–25.

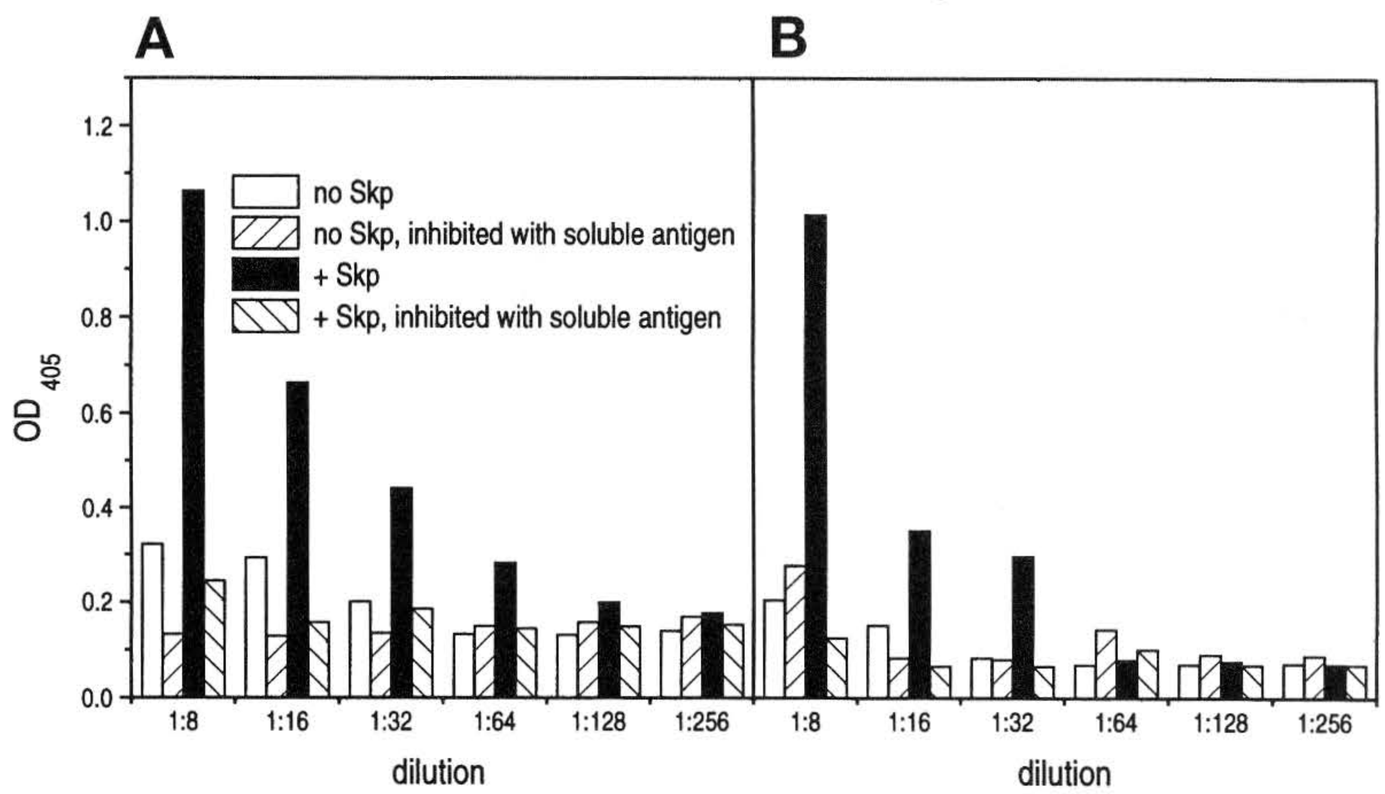

Figure 6. Crude extract ELISA. From *E. coli* JM83 expressing the soluble scFv fragments (A) 4-4-20 and (B) ABPC48-C(H22)S[23,24] with or without Skp crude extracts.

Discussion

We have identified a single gene of *E. coli* whose overexpression increases the amount of fusion proteins incorporated into phage. Because this effect is most dramatic on proteins that are aggregation-prone, explanations involving a general change of the expression level of the fusion protein or a general down-regulation of g3p wt can be ruled out. We previously found[17] that an increase in total fusion protein expression (by eliminating the suppressed stop codon at the junction between the scFv and g3p by a sense codon) leads to lower phage titers when a poorly folding scFv is used. Such phage are actually selected against, when challenged with the same construct containing a suppressible stop codon[17], presumably because high-expression phage create more stress in the host due to unfolded protein. Thus, unfolded fusion protein appears to be poorly incorporated and Skp appears to act by increasing the amount of correctly folded fusion protein, which then can get incorporated into the phage coat.

Skp is a very basic protein, which at first led to its misassignment as a DNA-binding protein[26] and later as an outer membrane–associated protein[27–29]. Homologs have been found in *Salmonella typhimurium*[28,29], *Yersinia enterocolitica*[30], *Yersinia pseudotuberculosis*[31], *H. influenzae*[32] and *P. multocida*[33]. Müller and co-workers[34] showed that this protein stimulates the in vitro import of *E. coli* proteins into membrane vesicles and subsequently established its periplasmic location[35], consistent with its soluble nature and the presence of a signal sequence. More recently, it has been proposed to be involved in the transport of outer-membrane proteins[16], and when its promoter region was interrupted by a Tn10 transposon, the heat shock factor σ^E (σ^{24})–dependent response was induced[6]. It remains unclear whether this is an effect of the absence of Skp or a polar effect on other proteins located downstream of *skp*. The heat shock response is probably induced indirectly via a change in the concentration of outer-membrane proteins, which is known to induce σ^E (σ^{24}) (ref. 6).

The genomic location of the *skp* gene suggests that it is involved in the transport of lipopolysaccharide (LPS), the major constituent of the *E. coli* outer membrane[36]. The LPS-binding properties of Skp have long been recognized[37]. *Skp* is the first gene of an operon[38] con-

taining one other gene, *lpxD (firA)*, which has been identified as UDP-3-O-[3-hydroxymyristoyl]-glucosamine-N-acyltransferase, a cytoplasmic enzyme involved in the biosynthesis of lipid A, a precursor of LPS. Separated by an intergenic region of 105 bp, the next operon downstream contains the genes *fabZ*, *lpxA*, *lpxB*, and *rnhA*, which code for (3R)-hydroxymyristoyl acyl-carrier-protein dehydrase, acyl-[acyl-carrier-protein]-UDP-N-acetylglucosamine-O-acyltransferase, lipid-A-disaccharide synthase, and ribonuclease HII[36]. The genes *fabZ*, *lpxA* and *lpxB* are also involved in fatty acid and lipid A biosynthesis[36]. The periplasmic location, high isoelectric point, and co-regulation with a lipid A synthetic enzyme would be consistent with a role of Skp in transporting the highly anionic LPS or a precursor to its final destination. As it is entirely possible and even likely that LPS and outer membrane proteins interact during their journey, Skp would also be expected to have an effect on outer-membrane protein transport[16].

Skp may be functionally defined as a molecular chaperone in that it leads to an increase in the amount of folded protein; however, we have no evidence yet of a direct involvement in protein folding or transient complex formation with the recombinant protein. Thus, it is entirely possible that Skp regulates the concentration of LPS or its precursors and prevents harmful interactions between some recombinant proteins and lipid A or other outer-membrane components. Alternatively, its effect may be more indirect, in promoting export of another factor that is titrated out by the poorly folding recombinant protein. A direct role is suggested as it was the only gene identified in the selection.

There are two transduction systems for periplasmic stress[7–10]. In the Cpx regulon[7], the inner membrane sensor kinase CpxA causes phosphorylation of its cognate response regulator CpxR and thus induces expression of DsbA, PpiA (RotA), and DegP. Intriguingly, the putative CpxR binding site[7] is also found upstream of *rpoE* (σ^E), *rpoH* (σ^{32}), *groE*, and *skp*. This makes the involvement of Skp in stress related to periplasmic folding problems very plausible. Other periplasmic factors involved in protein folding, such as FkpA and DegP, are under the control of σ^E, which receives its signal about periplasmic stress via the inner membrane protein RseA and possibly RseC and the periplasmic RseB[7–10]. There are at least two mechanisms by which poorly folding recombinant molecules (either periplasmically expressed or as membrane protein fusion to g3p) can be sensed, and Skp is one of the possible effector molecules.

Experimental protocol

Construction of genomic library. A NotI site was inserted in the phage display vector pAK100 (ref. 17) at position 5656. A polylinker was inserted as an oligonucleotide cassette into this NotI site. The gel-purified SfiI fragment encoding the scFv fragment of the antifluorescein antibody 4-4-20 (ref. 15) was ligated in this vector pHB100, yielding the plasmid pHB102. Genomic DNA of *E. coli* JM83 was isolated with Qiagen-tip 100G according to the manufacturer's protocol. The genomic DNA was partially digested with Sau3AI and applied to a 1% agarose gel. The range of 1 kb to 6 kb length was cut out, and the genomic DNA was eluted with GenElute agarose spin columns (Supelco, Bellefonte, PA), phenol/chloroform extracted and ethanol precipitated. After ligation of the *E. coli* library in the BglII site of the polylinker of pHB102, the ligation mixture was precipitated with n-butanol and electroporated into *E. coli* XL1-Blue (Stratagene, Basel, Switzerland). After plating on 2xYT in 530 cm^2 dishes (Nunc, Roskilde, Denmark) and overnight incubation at 37°C, the colonies were washed off the plates with 5 ml 2xYT, the OD_{550} was determined, and the cells were stored at -80°C after addition of glycerol to 10% final concentration.

Phage panning. A 10-ml culture of 2xYT, containing 15 μg/ml tetracycline (tet), containing 0.1 ml salt mixture (0.86 M NaCl, 0.25 M KCl, 1 M $MgCl_2$) was inoculated to an OD_{550} of 0.1 with *E. coli* harboring the genomic library. After 1 h incubation at 37°C chloramphenicol (cam) was added to a concentration of 30 μg/ml, and the cells were grown to an OD_{550} of 0.5. Then 10^{12} pfu of helper phage VCS M13 (Stratagene) were added and incubated for 15 min without agitation at 37°C, followed by addition of 50 ml 2xYT medium containing 30 μg/ml cam, 15 μg/ml tet, 0.5 ml salt mixture, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). The mixture was then shaken for 2 h at 37°C. After addition of 30 μg/ml kanamycin (kan), the cultures were grown overnight at 37°C. The cells were harvested and the phagemid DNA isolated (QIAprep spin kit; Qiagen, Basel, Switzerland). The phage from the culture supernatant were precipitated by incubation for 30 min with one-fourth volume PEG/NaCl solution (17% PEG 6000, 3.3 M NaCl, 1 mM EDTA) on ice, and the pellets were dissolved in 2 ml PBS (8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4 [ref. 39]). Immunotubes (Nunc) were coated with 20 μg/ml fluorescein-isothiocyanate coupled to bovine serum albumin (FITC-BSA) in PBS overnight at 4°C and blocked with 5% skimmed milk in PBST (PBS containing 0.05% Tween-20) for at least 1 h at room temperature. Five hundred microliters of the phage solution was filled to a final volume of 5 ml with 2% skimmed milk in PBST and applied to the tubes for 2 h at room temperature. The tubes were washed 20 times with PBST and twice with PBS. Bound phage were eluted with 1 ml 0.1 M glycine/HCl pH 2.2 for 10 min. The eluate was neutralized immediately with 60 μl 2 M Tris and the phages (typically 10^4–10^6 cfu) were used for reinfection.

Phage purification and ELISA. Phage ELISAs were carried out to assay the amount of functionally displayed scFv on M13 phage. Single colonies were grown at 37°C overnight in 5 ml 2xYT medium containing 30 μg/ml cam and 15 μg/ml tet. Ten milliliters of 2xYT medium containing 30 μg/ml cam, 15 μg/ml tet, 0.4% glucose, and 0.1 ml salt mixture were inoculated with the overnight culture to give an OD_{550} of 0.1. At an OD_{550} of 0.3 to 0.5, 10^{12} cfu VCS helper phage (Stratagene) were added. After 15 min, 50 ml 2xYT medium containing 30 μg/ml cam, 15 μg/ml tet, 0.5 ml salt mixture, and 0.1 mM IPTG was added. After 2 h at 37°C, kan was added to a final concentration of 30 μg/ml, and the cells were grown overnight. The phage were precipitated from the culture supernatant by incubating for 30 min with 1/4 volume PEG/NaCl solution as above on ice, and the pellets were redissolved in 1 ml PBS. After addition of 1.6 g of CsCl, the volume was adjusted to 4 ml with PBS. The CsCl solution was transferred into a 1/2 x 1 1/2 in. polyallomer tube and centrifuged at 100,000 r.p.m. for 4 h in a TLN-100 rotor (Beckman Instruments) at 4°C. After centrifugation the phage band was removed as described[40]. The phage were transferred to one-half by two inch polycarbonate tubes, which were filled with PBS to 3 ml. After centrifugation at 50,000 r.p.m. for 1 h in a TLA-100.3 rotor at 4°C, the pelleted phages were redissolved in 3 ml PBS. After an additional centrifugation at 50,000 r.p.m. for 1 h in a TLA-100.3 rotor at 4°C, the phage were dissolved in PBS. The concentration of phage particles was quantified spectrophotometrically[41]. ELISA plates were coated with FITC-BSA in PBS, for antilevan antibodies with 10 μg/ml levan (polyfructose, Sigma) in PBS at 4°C overnight. The plates were blocked for 1 h at room temperature. A defined number of purified phage (measured[41] by OD) was mixed with 2% skimmed milk in PBST in the absence and presence of 10 μM fluorescein or 0.05% levan and applied to the blocked ELISA plates and incubated for 1 h at room temperature. Detection was as above, using an anti-M13 antibody conjugated with horseradish peroxidase (Pharmacia, Dübendorf, Switzerland).

Phage blots. 10^{11} phage were applied to a reducing 11% SDS-PAGE, and blotted on nitrocellulose membranes. Detection was carried out with the monoclonal antibody 10C3 (ref. 19), which recognizes the C-terminal half of g3p (1:50,000 in TBST (25 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 2% skimmed milk), for 60 min at room temperature, followed by incubation with a polyclonal antimouse-peroxidase conjugate (Pierce, Rockford, IL) (1:5000 in TBST/2% milk, 45 min RT), and using the ECL-kit (Amersham, Zürich, Switzerland).

Crude extract ELISA. Fifty milliliters of LB medium containing 30 μg/ml cam were inoculated with a single colony of *E. coli* JM83, harboring a plasmid encoding the respective scFv fragment. The cultures were grown at 24°C to an OD_{550} of 0.5 and induced with 1 mM IPTG. After overnight induction at 24°C the cells were harvested and resuspended in 4 ml PBS. Whole cell extracts were prepared by French Press lysis at 10,000 psi and 1 ml of the crude extract was centrifuged in an Eppendorf tube for 30 min at 50,000 r.p.m. in a TLA-100.3 rotor (Beckman Instruments) at 4°C. After centrifugation, the supernatants containing the soluble material were normalized to an OD_{550} of 20 in 1 ml. ELISA plates were coated and blocked as described above for phage ELISAs. A defined amount of crude extract was mixed with 2% skimmed milk in PBST in the absense and presence of 10 μM fluorescein or 0.05% levan and applied to the blocked ELISA plates and incubated for 1 h at room temperature. The signal was detected with an anti-myc-tag antibody[42] and an antimouse antibody conjugated with horseradish peroxidase and soluble BM blue POD-substrate (Boehringer Mannheim, Rotkreuz, Switzerland), and

after stopping the reaction with 0.1 M HCl, the signals were read at 405 nm.

Protein purification. The antiphosphorylcholine scFv McPC603-H11 (ref. 20) was purified using affinity chromatography[43] prepared as described[43] in the presence and absence of coexpressed Skp. The concentration and yield was estimated photometrically using a calculated extinction coefficient[44].

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