THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 22, Issue of June 2, pp. 17100–17105, 2000

Printed in U.S.A.

The Periplasmic *Escherichia coli* Peptidylprolyl *cis,trans*-Isomerase FkpA

I. INCREASED FUNCTIONAL EXPRESSION OF ANTIBODY FRAGMENTS WITH AND WITHOUT *cis*-PROLINES*

Received for publication, December 23, 1999, and in revised form, March 8, 2000
Published, JBC Papers in Press, March 22, 2000, DOI 10.1074/jbc.M910233199

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The production of recombinant proteins in the periplasm of *Escherichia coli* can be limited by folding problems, leading to periplasmic aggregates. We used a selection system for periplasmic chaperones based on the coexpression of an *E. coli* library with a poorly expressing antibody single-chain Fv (scFv) fragment displayed on filamentous phage (Bothmann, H., and Plückthun, A. (1998) *Nature Biotechnol.* 16, 376–380). By selection for a functional antibody, the protein Skp had been enriched previously and shown to improve periplasmic expression of a wide range of scFv fragments. This selection strategy was now repeated with a library constructed from the genomic DNA of an *skp*-deficient strain, leading to enrichment of the periplasmic peptidylprolyl *cis,trans*-isomerase (PPIase) FkpA. Coexpression of FkpA increased the amount of fusion protein displayed on the phage and dramatically improved functional periplasmic expression even of scFv fragments not containing *cis*-prolines. In contrast, the coexpression of the periplasmic PPIases PpiA and SurA showed no increase in the functional scFv fragment level in the periplasm or displayed on phage. Together with the *in vitro* data in the accompanying paper (Ramm, K., and Plückthun, A. (2000) *J. Biol. Chem.* 275, 17106–17113), we conclude that the effect of FkpA is independent of its PPIase activity.

An important strategy for the production of recombinant disulfide-containing proteins is their secretion to the periplasmic space of *Escherichia coli*, as the periplasm is the location of the disulfide-forming machinery (1, 2). Antibodies are one class of particularly important disulfide-containing proteins that have frequently been expressed in this compartment, and this is probably the most convenient method to obtain engineered antibodies for research and medical or technological applications (3–5). Unfortunately, folding problems of antibodies and other heterologous proteins in the periplasm are frequently observed, and the nature and existence of molecular chaperones in this compartment are still only poorly understood (2, 6, 7).

It has been previously shown (8–12) that the functional periplasmic expression of antibody fragments can be limited by the sequence-dependent, moderate efficiency of periplasmic folding, which can furthermore be accompanied by cell lysis, as has also been observed for other proteins (13). Typically, the actual membrane transport is not limiting (8), and large amounts of correctly processed, but precipitated protein are available in the periplasm. It is still unclear which factors, if any, might play a role in preventing the aggregation and in guiding the correct folding of functional antibody fragments or other periplasmic proteins in *E. coli*, and it is certainly conceivable that different factors play a role for different proteins.

We have recently developed a selection system for the identification of novel factors that may help the functional periplasmic expression of a substrate protein in question (14). It was based on the assumption that the folding of soluble periplasmic protein occurs in contact with the same machinery and in the same environment as that of a protein displayed on filamentous phage. Fusion proteins with the minor coat protein gene-3 protein (g3p)[1] of filamentous phage are transiently produced in a state anchored to the inner membrane, before they are incorporated into the phage coat (15). Thus, the folding of the fusion protein occurs in the periplasm and should be influenced by the same factors as that of a soluble periplasmic protein.

The selection system works by displaying a poorly expressing antibody fragment on filamentous phage and coexpressing a library of *E. coli* genes on the same phagemid. Note that these *E. coli* proteins encoded by the library are not displayed; they are merely expressed by the host cell producing a particular phage in question. If the coexpressed factor improves expression of the antibody-g3p fusion protein, a higher percentage of the phage particles will carry a functional antibody fragment and thus be selectable, even though this displayed antibody fragment is identical in all cases.

In a previous study, we identified the periplasmic protein Skp (OmpH, HlpA) by this methodology (14). Since we wanted to select for additional factors that might increase the expression yield, we have repeated this selection in the present study, this time using an *E. coli* library prepared from a strain devoid of the *skp* gene. We enriched the gene coding for the protein FkpA, a periplasmic peptidylprolyl *cis,trans*-isomerase (16). Upon characterizing the effect further, we found that only FkpA, but not the other periplasmic peptidylprolyl *cis,trans*-isomerases PpiA and SurA, had any such effect, suggesting that there is no functional redundancy in these enzymes. Most intriguingly, we found that FkpA has also a very beneficial effect on antibody fragments whose rate-limiting proline

* This work was supported by Schweizerischer Nationalfonds Grants 31-46624.96 and 31-47302.96. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: g3p, gene-3 protein; scFv, single-chain Fv; kb, kilobase pair(s); FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s); PCR, polymerase chain reaction; PPIase, peptidylprolyl *cis,trans*-isomerase; FKBP, FK506-binding protein.

isomerization it cannot catalyze and, particularly noteworthy, that do not have any *cis*-proline at all.

EXPERIMENTAL PROCEDURES

Construction of Genomic Library—The gel-purified *Sfi*I fragment encoding the scFv fragment of the anti-levan antibody ABPC48-C(H22)S (17, 18) was ligated into the phage display vector pHB100 (14), yielding plasmid pHB121. The genomic DNA of the *skp*-deficient *E. coli* strain RC354c (19) was isolated with a Nucleobond AXG100 cartridge (Macherey Nagel) according to the manufacturer's protocol. The genomic DNA was partially digested with *Sau*3AI and applied to a 1% agarose gel. A range of fragment sizes from 1 to 6 kb in length was cut out, and the genomic DNA was eluted with GenElute™ agarose spin columns (Supelco Inc.), phenol/chloroform-extracted, and ethanol-precipitated. After ligation of the *E. coli* library into the *Bgl*II site of the polylinker of pHB121, the ligation mixture was precipitated with 1-butanol and electroporated into *E. coli* XL1-Blue (Stratagene). After plating on 2× YT in 530-cm^2 dishes (Nunc) and overnight incubation at 37 °C, the colonies were washed off the plates with 5 ml of 2× YT; OD_{550} was determined; and the cells were stored at −80 °C after addition of glycerol to 50% final concentration.

Phage Panning—Phage panning was carried out as described (14). For enriching the levan-binding scFv fragment, immunotubes (Nunc) were coated with 10 μg/ml levan (polyfructose; Sigma) in phosphate-buffered saline (8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 3 mM KCl, pH 7.4) overnight at 4 °C, and for enriching anti-fluorescein antibodies, with 20 mg/ml fluorescein isothiocyanate coupled to bovine serum albumin in phosphate-buffered saline and blocked with 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20 for at least 1 h at room temperature.

Phage Purification and ELISA—Phage purification and ELISA were carried out exactly as described (14).

Western Blots—To compare the *in vivo* folding properties of the scFv fragments with and without coexpressed factors, the ratio of soluble to insoluble material that had accumulated in the periplasm during expression was determined. A 50-ml volume of LB medium containing 30 μg/ml chloramphenicol was inoculated to OD_{550} = 0.1 with an overnight culture, grown at 37 °C from a single colony of *E. coli* JM83, harboring a plasmid encoding the respective antibody fragment. The cultures were grown at 24 or 37 °C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside at OD_{550} = 0.5. After 3 h of induction at 37 °C or overnight induction at 24 °C, the cells were harvested and resuspended in 4 ml of phosphate-buffered saline. Whole cell extracts were prepared by French press lysis at 10,000 p.s.i., and 1 ml of the crude extract was centrifuged in an Eppendorf tube for 30 min at 50,000 rpm in a TLA-100.3 rotor (Beckman Instruments) at 4 °C. After centrifugation, the supernatants contained the soluble material. The pellets were dissolved in urea-containing buffer as described previously (10). Both fractions were normalized according to the OD_{550} and analyzed by reducing SDS-polyacrylamide gel electrophoresis with subsequent Western blotting on a nitrocellulose membrane. Immunostaining was performed with the anti-FLAG antibody M1 (Eastman Kodak Co.) as the primary antibody (20) and an Fc-specific anti-mouse antiserum conjugated to horseradish peroxidase (Pierce) as the secondary antibody. For chemiluminescent detection, the SuperSignal kit (Pierce) was used.

Crude Extract ELISA—Preparation of *E. coli* extracts and ELISA were carried out as described (14). For cultures grown at 37 °C, the cells were harvested 3 h after induction.

RESULTS

Phage Selection and Identification of Coexpressed Factors—We used a phagemid displaying the poorly folding scFv fragment of the anti-levan antibody ABPC48-C(H22)S as the recipient for an *E. coli* genomic library. The genomic DNA of *E. coli* RC354c, an *skp*-deficient strain (19), was size-fractionated from 1 to 6 kb and ligated into the polylinker of plasmid pHB121 (see "Experimental Procedures"). Thus, *E. coli* genes, regulated under their own promoters, are overexpressed on the phagemid, primarily through an effect of vector copy number. A library size of 6.4×10^5 clones ensured that each piece of the *E. coli* genome should be represented, provided it led to viable clones.

Seven panning rounds on levan were carried out, and phagemid DNA from each round was cut with the restriction enzyme *Not*I to detect the accumulation of any inserts. It can be seen in Fig. 1 that two bands of ~1.7 and 2.0 kb accumulated throughout the panning. Fourteen of 17 single colonies analyzed after the seventh round carried the 1.7-kb insert, and three carried the 2.0-kb insert. Both inserts were sequenced (1629 and 1987 bp, respectively) and were found to contain the same two complete genes coding for the periplasmic protein FkpA (Fig. 2) (16, 21) and the open reading frame SlyX with unknown function. Both fragments end 218 bp after the stop codon of *fkpA*. Therefore, both inserts also contain the first 21 amino acids of the gene *yheO* (Swiss-Prot accession number P45533), which codes for a protein with unknown function and strong similarity to *Hemophilus influenzae* HI0575. The 1629-bp insert continues for 159 bp downstream of the stop codon of *slyX*, whereas the 1987-bp insert continues for 528 bp downstream of the stop codon of *slyX*. Both inserts therefore also contain the C-terminal part of *slyD* (WHP) (22–25). The 1987-bp insert also contains the first 117 amino acids of YheP, an open reading frame with unknown function or significance.

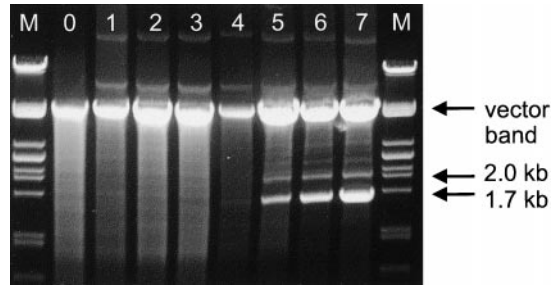

FIG. 1. **Analysis of phagemid pools after different panning rounds.** The phagemid pools were analyzed by restriction enzyme digest with *Not*I. *Lanes M*, *Pst*I-digested λ-DNA as molecular weight marker; *lane 0*, phagemids before first panning round; *lanes 1–7*, panning rounds from which phagemids were isolated.

The panning procedure was also carried out with the fluorescein-binding scFv fragment 4-4-20 (26–28) on FITC-bovine serum albumin. The library size was 6.9×10^5, and 5 of 10 single colonies analyzed after the seventh panning round showed the same 2.0-kb insert as enriched with the anti-levan antibody (data not shown). One clone showed a 1.8-kb insert. This band was sequenced and contained a 1650-bp insert, starting 120 bp downstream of the stop codon of *slyX* and ending 278 bp downstream of the stop codon of *fkpA* (Fig. 2).

To examine which of the two complete genes present on the fragments, obtained by panning with two different antibodies, is responsible for the enrichment, *fkpA* and *slyX* were PCR-amplified and recloned separately as well as together at the same position in vectors pHB102 and pHB121, which display the FITC-bovine serum albumin-binding scFv fragment 4-4-20 and the levan-binding scFv fragment ABPC48-C(H22)S, respectively (14). The *fkpA* gene was PCR-amplified from the translation start site of *slyX* to the stop codon of *fkpA* (Fig. 2) to ensure that it contains the whole *fkpA* upstream region. Conversely, the *slyX* gene was amplified between the translation start site of *fkpA* and the stop codon of *slyX* for the same reason. The DNA fragment containing both genes was PCR-amplified between the stop codons of *fkpA* and *slyX*. The PCR primers contained *Not*I sites at their ends, and the fragments were cloned in the corresponding site in pHB102 and pHB121.

Characterization of the Influence of FkpA and SlyX on Phage Display and Soluble Expression—For examination of the possible effects of FkpA on the yield of periplasmically expressed proteins, several different scFv fragments were chosen as test molecules for the following reasons. The 4-4-20 scFv fragment (27) and the ABPC48-C(H22)S scFv fragment (17, 18) show rather poor folding properties as compared with the well expressed FITC-E2 scFv fragment (29, 30). The scFv fragment

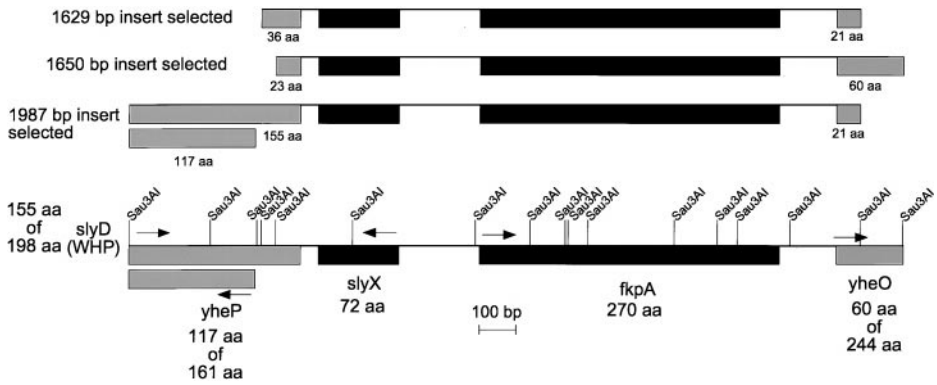

FIG. 2. **Schematic representation of the 1629-, 1650-, and 1987-bp fragments enriched by phage display and phage panning.** YheO is the putative product of an open reading frame with unknown function, but with strong similarity to *H. influenzae* HI0575. *fkpA* and *slyD* code for peptidylprolyl *cis,trans*-isomerases; *slyX* and *yheP* code for proteins with unknown function. *Arrows* indicate orientation of the open reading frames. *aa*, amino acids.

McPC603-H11 (10) has been a model system in previous studies (9, 10) and can be easily quantitated on a large scale. The disulfide-free hu4D5-8 scFv fragment (abbreviated 4D5^{--}, to indicate the missing disulfide bonds) (31) is thermodynamically unstable. Finally, the anti-GCN4 scFv fragment carries a λ-chain (32) and thus no *cis*-prolines, and a destabilized variant with the H-R66K mutation was chosen (33) where H refers to the heavy chain, to observe a possible improvement in folding. Furthermore, the folding of the last three antibodies has been characterized *in vitro* (34–36).

To determine how and why FkpA, or possibly SlyX, became enriched, we characterized the phage produced in the absence and presence of coexpressed FkpA and/or SlyX. The antigen-binding phage ELISA (Fig. 3*a*) showed that overexpression of FkpA significantly increased the number of functional antibody molecules on the phage in the case of scFv fragment 4-4-20 compared with the control phage not overexpressing an additional factor and with those that overexpress Skp. In contrast, the overexpression of SlyX had no effect on the number of functional antibody molecules on the phage. Skp and FkpA expressed together had only a very slightly higher effect than FkpA alone.

For scFv fragment ABPC48-C(H22)S (Fig. 3*b*), the influence of FkpA was somewhat smaller compared with that of Skp. Again, no influence of SlyX could be detected. The coexpression of FkpA and Skp had no significant additional benefit compared with FkpA alone. In the case of the well produced antibody FITC-E2 (Fig. 3*c*), the coexpression of FkpA also had no significant effect on the ELISA signal, whereas the coexpression of SkpA resulted in somewhat more functional scFv fragment displayed on phage.

We then determined whether the beneficial effect on phage ELISA, indicating functional scFv-g3p fusion protein, is also reflected in the total amount of fusion protein per phage. For this purpose, we analyzed by Western blotting the amount of full-length fusion protein on CsCl-purified phage particles in the presence and absence of *fkpA* and *slyX* on the phagemid using the monoclonal antibody 10C3 (37), which recognizes the C-terminal domain of g3p (Fig. 3, *d* and *e*). For scFv fragment 4-4-20, the coexpression of FkpA dramatically increased the amount of fusion protein on the phage. This effect was higher than for the coexpression of Skp, whereas the coexpression of both genes led to a small further increase in incorporated fusion protein. A positive effect of the coexpression of SlyX was not detectable. For scFv fragments ABPC48-C(H22)S, FITC-E2, and McPC603-H11 (Fig. 3, *d* and *e*), the coexpression of FkpA had a positive effect compared with the absence of coexpression, but the benefit was significantly smaller than the coexpression of Skp for scFv fragments ABPC48-C(H22)S and FITC-E2. For McPC603-H11, the influence of Skp and FkpA was about equal.

We then examined the effect of FkpA on the soluble periplasmic expression of the disulfide-free scFv 4D5^{--} fragment at 24 °C. The amount of soluble scFv fragment was >10-fold increased in the presence of FkpA compared with its absence (Fig. 4*a*). The corresponding Western blot shows a dramatic increase in soluble material if FkpA was overexpressed. This antibody had been shown not to be amenable to rate acceleration by PpiA[2] or FkpA (35) even though the proline *cis,trans*-isomerization is rate-limiting (34).

Coexpression of PpiA and SurA—Since out of three soluble peptidylprolyl *cis,trans*-isomerases in the periplasm (PpiA, SurA, and FkpA), only FkpA became enriched during the panning procedure, we wanted to investigate the possible influence of PpiA and SurA in our model system. Furthermore, we were interested to determine if the expression of the putative folding catalysts FkpA, PpiA, and SurA is induced by the expression and subsequent accumulation of poorly folding scFv fragments, as periplasmic folding problems may turn on a stress response (see below). We thus PCR-cloned PpiA and SurA with their own promoters and inserted them at the same position as FkpA in the vector pHB102. We then examined the effect of FkpA, PpiA, and SurA on the production of the 4-4-20 scFv fragment in soluble form using the nonsuppressor strain JM83 at 24 °C (Fig. 5*a*). The amount of soluble scFv fragment 4-4-20 was dramatically increased in the presence of overexpressed FkpA compared with its absence. In contrast, the coexpression of PpiA and SurA showed no improvement of soluble material. Western blots of the soluble and insoluble proteins of the scFv fragments confirm these results (Fig. 5*b*).

The overexpression of FkpA and PpiA was tested by Western blotting with the aid of a recombinant anti-His tag antibody (38), as they had been PCR-cloned with a histidine tag (data not shown). We saw no significant difference in the expression levels of FkpA and PpiA whether the scFv fragment was induced or not. Since SurA could not be detected by Western blotting, we conclude that its expression level is much lower than those of FkpA and PpiA under these conditions (data not shown).

To overcome possible regulatory effects, we also cloned the three genes *fkpA*, *ppiA*, and *surA* each separately in a direct operon fusion behind scFv fragments 4-4-20 and ABPC48-C(H22)S. Therefore, they were expressed together with the scFv fragment by induction with isopropyl-β-D-thiogalactopyranoside. After expression at 24 °C overnight, all three coexpressed proteins could be detected by Western blotting with the anti-His tag antibody and as additional bands on SDS-polyacrylamide gel stained by Coomassie Blue (data not shown). Also under these conditions, no improvement in the amount of soluble scFv was obtained by the coexpression of PpiA and SurA (Fig. 6, *c* and *d*). Expression experiments carried out at

[2] M. Jäger and A. Plückthun, unpublished results.

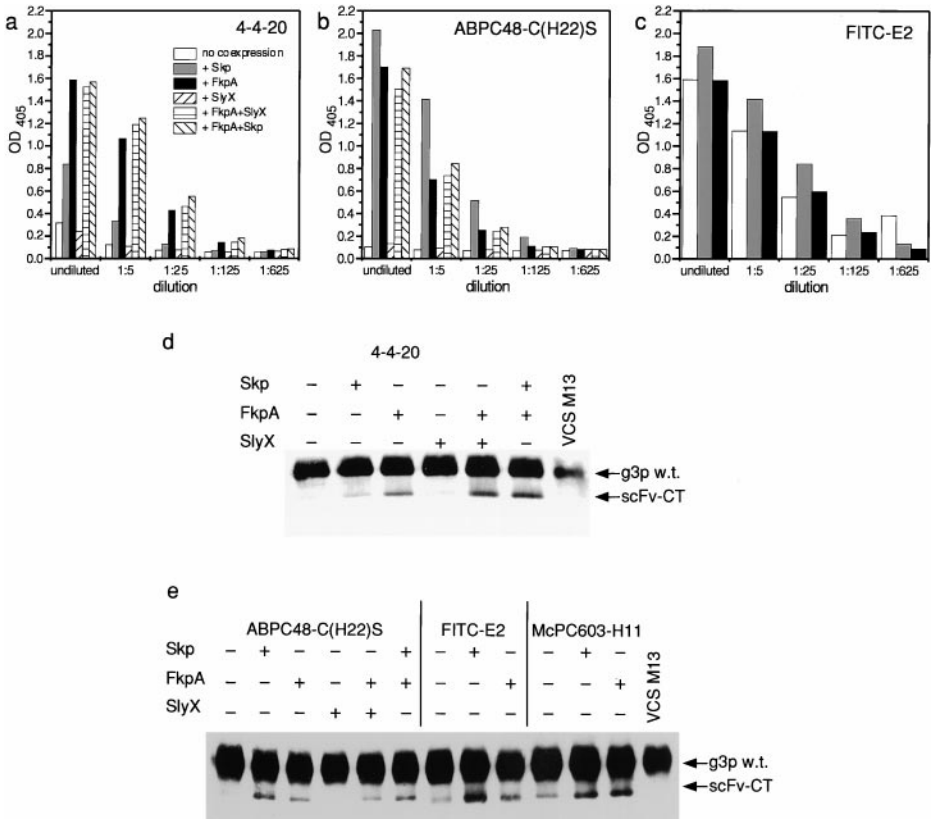

FIG. 3. *a–c*, antigen-binding ELISAs of phages grown with or without overexpressed Skp, FkpA, SlyX, and combinations of them, displaying scFv fragments 4-4-20 (27), ABPC48-C(H22)S (17, 18), and FITC-E2 (29, 30), respectively. Phages were purified by CsCl gradients. *d* and *e*, phage blots. Phage carrying fusions of the scFv fragments to the C-terminal domain (*CT*) of g3p of the antibodies indicated were grown with or without overexpression of Skp, FkpA, SlyX, and combinations of them. Phages were purified by CsCl gradients. Helper phage VCS M13 was used as size reference for wild-type (*w.t.*) g3p. Since the scFv fragment is fused to the C-terminal domain of g3p, it runs below wild-type g3p on SDS-polyacrylamide gel. The blot was developed with antibody 10C3 (37). The different scFv phages have been described (10, 17, 18, 27, 29, 30) (C. Freund, unpublished results).

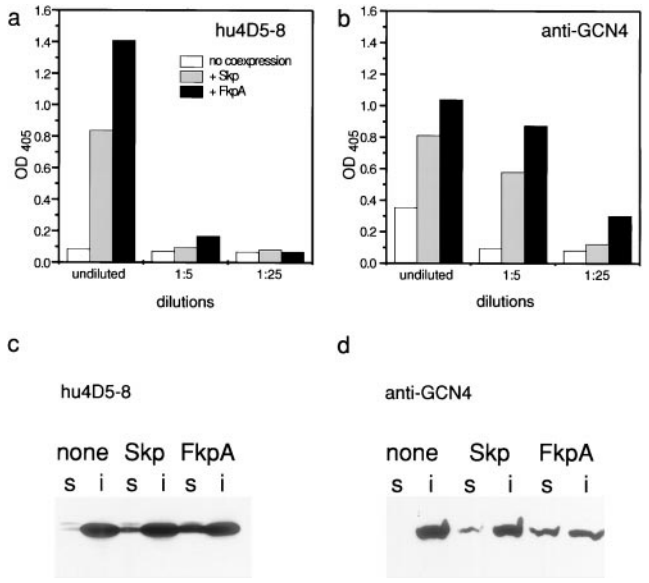

FIG. 4. **Antigen-binding ELISA (*a* and *b*) and Western blotting (*c* and *d*) of crude extracts of *E. coli* JM83 expressing the soluble scFv fragments 4D5^{-} $^{-}$ (*a* and *c*) (31, 34) at 24 °C and anti-GCN4 (H-R66K) (*b* and *d*) (33) at 37 °C with or without Skp and FkpA.** *i*, insoluble material; *s*, soluble material.

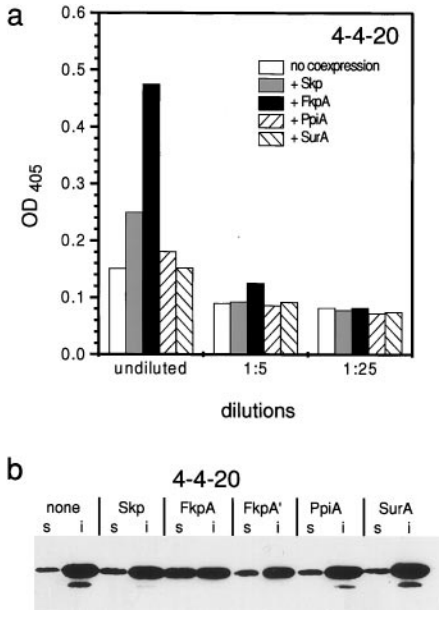

FIG. 5. **Antigen-binding ELISA (*a*) and Western blotting (*b*) of crude extracts of *E. coli* JM83 expressing the soluble scFv fragment 4-4-20 (27) with or without Skp, FkpA, PpiA, and SurA.** FkpA′ is missing the last 123 amino acids of FkpA. *i*, insoluble material; *s*, soluble material.

37 °C showed the same results. The improvement in periplasmic folding was caused only by FkpA and the previously described Skp. Again, the results from the ELISA experiments, measuring functional protein, were also reflected in the amount of soluble protein (Fig. 6, *a* and *b*). It can clearly be seen that only FkpA dramatically improved the amount of scFv fragment 4-4-20, and it had the greatest effect on ABPC48-C(H22)S, for which, however, also Skp was beneficial, and perhaps a very slight effect of PpiA and SurA cannot be excluded.

Influence of FkpA on in Vivo Expression of an scFv Fragment Missing cis-Prolines—To further characterize the function of FkpA, we studied the anti-GCN4 scFv fragment with the destabilizing mutation H-R66K (33). This scFv fragment carries a murine λ-chain derived from the gene coding for variable region $V_{\lambda}1$. The mouse λ locus contains three genes coding for variable regions $V_{\lambda}1$, $V_{\lambda}2$, and $V_{\lambda}X$, of which $V_{\lambda}1$ and $V_{\lambda}2$ are closely related, whereas the $V_{\lambda}X$ amino acid sequence is as divergent to other V_{λ} genes sequences as to V_{κ} gene sequences

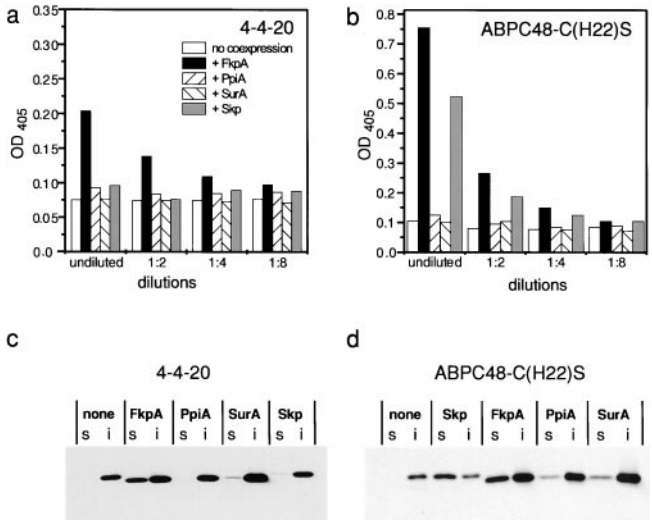

FIG. 6. **Antigen-binding ELISA (*a* and *b*) and Western blotting (*c* and *d*) of crude extracts of *E. coli* JM83 expressing the soluble scFv fragments 4-4-20 (*a* and *c*) (27) and ABPC48-C(H22)S (*b* and *d*) (17, 18) with or without Skp, FkpA, PpiA, and SurA at 37 °C.** *i*, insoluble material; *s*, soluble material.

(39). Antibodies derived from the $V_\lambda 1$ gene, as is the case for the anti-GCN4 scFv fragment, have been crystallized in at least 22 independent crystal structures, and in no case has a *cis*-proline been observed. Also, V_H domains never have *cis*-prolines, as found in 205 independent structures.[3] Therefore, the folding of this scFv fragment is not proline-limited.

We examined the effect of FkpA on the soluble periplasmic production of the anti-GCN4 scFv fragment at 37 °C. A significant influence of coexpressed FkpA could be observed. The amount of soluble material in crude extract ELISA and Western blotting was significantly increased (Fig. 4, *b* and *d*), even though there was no need for a PPIase function on this scFv fragment to reach a functional state.

DISCUSSION

In this work, we validated our selection system for periplasmic folding factors. They may be extremely useful in increasing the functional yield of recombinant proteins such as antibodies, which need to be produced in the periplasm because of their disulfide bonds. We believe that this selection approach is very promising for the identification of additional factors involved in periplasmic folding and in preventing misfolding and that this strategy can be expanded to the screening of different genetic libraries for such factors. The protein FkpA, which we have enriched, indeed clearly improves the expression yield of a wide range of functional scFv fragments in the periplasm of *E. coli* (in some cases, 10-fold). This greatly beneficial effect on the folding yield is visible for all scFv fragments tested, except one, which has good folding properties anyway.

FkpA was first described as an *E. coli* protein homologous to the eukaryotic FK506-binding proteins (FKBPs) (16), a class of well characterized PPIases that have been shown to be inhibitable by the macrolide FK506. Mature FkpA is located in the periplasm (21), and its gene carries σ^E-binding sites in the promoter region (40). FkpA shows a high peptidyl *cis,trans*-isomerase activity, as shown with RNase T1 as substrate (35).

Antibody Folding and Effect of FkpA—The *in vitro* folding of antibody scFv fragments containing κ light chains, which normally contain two *cis*-prolines (positions L8 and L95), has been investigated in detail (34, 36, 41, 42). The rate-limiting step is the *trans,cis*-isomerization of the conserved Pro-L95 in the variable κ domain, which is a necessary prerequisite for the correct docking of V_H. Human cyclophilin and *E. coli* PpiA have been shown to catalyze this reaction *in vitro* for the isolated V_L domain and the Fv fragment (where the domains are unlinked) (42), but inefficiently or even not at all for scFv fragments, probably because the domains are inaccessible in an early folding intermediate (36, 41, 42). The slow step of the scFv fragment of the antibody hu4D5-8, with or without disulfides, cannot be accelerated by PPIases (neither PpiA[2] nor FkpA) (35).

FkpA has been independently selected on two occasions with different antibodies. It is remarkable that it also dramatically improves the folding of an antibody whose rate-limiting step it cannot catalyze *in vitro* ($4D5^{--}$), and even more striking is that its coexpression increases the yield of an scFv fragment lacking *cis*-prolines. This suggests a folding assisting activity that is independent of the PPIase activity. This result is supported by the fact that neither PpiA nor SurA can replace FkpA in coexpression experiments. Previous experiments (9) in which PpiA was overexpressed together with the Fv, scFv, and Fab fragments of the antibody McPC603 also failed to show any significant effect for any of the fragments, consistent with the results found here for PpiA with a different series of scFv fragments.

A "chaperone-like" activity of PPIases has been discussed controversially before (43–45). The debate was fueled by the fact that the model system used, human carbonic anhydrase II, still contains two *cis*-prolines, requiring complicated analyses. Experiments with citrate synthase as substrate showed that FKBP52 possesses chaperone activity independently of its PPIase activity (46). Additionally, cyclophilin Cyp-40 shows chaperone activity on denatured β-galactosidase, which is not affected by cyclosporin A, suggesting that its chaperone activity is not dependent on peptidyl *cis,trans*-isomerase activity (47).

Function and Regulation of FkpA—In *E. coli*, all periplasmic activities of FkpA, PpiA, SurA, and PpiD have been individually deleted, and the cells remained viable, also in the double mutants *surA*/*rotA* and *fkpA*/*rotA* (21, 48, 49). Nevertheless, based on the effects seen with *surA* mutants, SurA has been proposed to be involved in outer membrane protein localization and folding (50, 51), as has PpiD (49). Interestingly, the *surA*/*ppiD* double mutant causes lethality, whereas the *surA* null mutant can be combined with *ppiA*, *fkpA*, or even an *skp* null mutant. No particular substrate protein has been proposed yet for PpiA or FkpA.

Although the deletion of FkpA is not lethal, it leads to increased σ^E activity (40). Together with the findings that high level synthesis of FkpA can suppress the accumulation of unfolded periplasmic and outer membrane proteins, it has been suggested that it acts as a global folding catalyst (21). The expression of FkpA is increased by overproduction of σ^E and by creation of extracytoplasmic stress; additionally, σ^E-binding sites are found in its promoter region (40).

Our experiments do not suggest that the physiological role of FkpA for *E. coli* periplasmic proteins is independent of its PPIase activity, which it unequivocally has (35). On the other hand, the repeated enrichment of FkpA in independent phage panning experiments shows the strong benefit of FkpA overexpression, which is more strikingly verified with model proteins not containing *cis*-prolines. Were the effect "unspecific," a wide variety of proteins should be able to improve antibody expression when overexpressed. This is, however, not the case.

It would be plausible to propose that the peptide-binding site of FkpA may be used to reversibly bind part of the antibody. Whether FkpA acts as a "protein buffer" (52); binds early in the pathway like the trigger factor, which is a ribosome-bound

[3] A. Honegger, personal communication.

FKBP (53–55); rescues proteins that have denatured from the native state (56); or does even all of the above remains the subject of further studies.

Multiple Functions of PPIases—The dual biological role of these classes of proteins, which, on the one hand, are able to catalyze the isomerization of a peptide bond and, on the other hand, are involved in signal transduction at least in some cells (57–61), has never been fully resolved. Particularly, it has remained mysterious why so many apparently redundant activities are present in most genomes. Moreover, attempts to delete one or several of these enzymes have rarely led to obvious phenotypic effects. In addition to the experiments performed with the deletion of the *E. coli* genes (see above), all 12 cyclophilin and FKBP genes of yeast, identified with the knowledge of the complete genome sequence, have been deleted, but the cells were still viable (62), whereas the deletion of the only parvulin homologue in yeast, *ESS1*, is lethal (63, 64). Similarly, two out of four PPIases have been deleted from *Bacillus subtilis*, with no phenotypic consequence in rich medium or under several stress conditions tested. The double mutant showed a strongly retarded growth behavior only in the absence of all amino acids except tryptophan and phenylalanine (65). In *Erwinia chrysanthemi*, disruption of *rotA* caused no change in cell morphology, cell viability, growth rate, or stability of the extracellular and periplasmic proteins (66). It is certainly possible that a subset of PPIases fulfills a more general binding function, which can be exploited to improve folding.

Conclusions—Taking all the facts together, we conclude that the beneficial effect of FkpA overexpression must be independent of proline *cis,trans*-isomerase activity. First, the effect is found only with FkpA, but not with PpiA or SurA; and second, it is found also with a model protein devoid of any *cis*-prolines. Additionally, a very strong effect is found with a protein ($4D5^{--}$) whose rate-limiting step cannot be accelerated by FkpA (35). These *in vivo* findings are directly reflected *in vitro*, where the presence of FkpA during folding improves the yield under conditions favoring aggregation, but does not accelerate the slow step (35).

Our results have also validated the selection strategy that uses phage display of a poorly expressible protein. It is likely that this approach can be repeated, even with libraries of genes from organisms other than *E. coli*. Finally, the overexpression of FkpA helps to solve a long-standing practical problem in antibody engineering by improving the expression of a wide variety of different antibodies tested.

Acknowledgments—We thank Ulrich Henning (Max-Planck-Institut for Biology, Tübingen, Germany) for the kind gift of bacterial strain RC354c and Kathrin Ramm, Sabine Jung, Jörg Burmester, Alain Tissot, and Jörg Willuda for helpful discussions.

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