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Escherichia coli trigger factor is a prolyl isomerase that associates with nascent polypeptide chains

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ABSTRACT **Correct folding of newly synthesized proteins is proposed to be assisted by molecular chaperones and folding catalysts. To identify cellular factors involved in the initial stages of this process we searched for proteins associated with nascent polypeptide chains. In an *Escherichia coli* transcription/translation system synthesizing β-galactosidase we identified a 58-kDa protein which associated with translating ribosomes but dissociated from these ribosomes upon release of nascent β-galactosidase. N-terminal sequencing identified it as trigger factor, previously implicated in protein secretion. Direct evidence for association of trigger factor with nascent polypeptide chains was obtained by crosslinking. In a wheat germ translation system complemented with *E. coli* lysates, ε-4-(3-trifluoromethyldiazirino)benzoic acid-lysine residues were incorporated into nascent secretory preprolactin and a nonsecretory preprolactin mutant. Trigger factor crosslinked to both types of nascent chains, provided they were ribosome bound. Trigger factor contains key residues of the substrate-binding pocket of FK506-binding protein-type peptidyl-prolyl-cis/trans-isomerases and has prolyl isomerase activity *in vitro*. We propose that trigger factor is a folding catalyst acting cotranslationally.**

The high efficiency of *in vivo* folding of newly synthesized proteins to their native structures is particularly intriguing in view of the difficulties that this process has to overcome. First, translation generates nascent polypeptide chains transiently containing incomplete folding information. Second, nascent chains are synthesized in a milieu containing high concentrations of aggregation-prone folding intermediates (1). Productive folding might therefore require a mechanism to delay folding until the required folding information is available and to shield the nascent chain from other folding proteins. Third, the rapidity of folding can best be explained if slow folding steps, including isomerization of peptidyl-prolyl peptide bonds and formation of disulfide bonds, are catalyzed. The discovery of folding catalysts, including peptidyl-prolyl-cis/trans-isomerases (PPIases) and protein disulfide isomerases (2–4), and molecular chaperones, including the DnaK/Hsp70 and GroEL/Hsp60 chaperone systems (1, 2), is in support of the hypothesis that folding of newly synthesized proteins is an assisted process.

There is only limited experimental evidence for the participation of chaperones and folding catalysts in folding of newly synthesized polypeptides. Association with nascent polypeptides has been reported for mammalian Hsp72/73 (5), and the Ssb1/2p members of the Hsp70 family in *Saccharomyces cerevisiae* (6). The *Escherichia coli* chaperone DnaJ was crosslinked to nascent firefly luciferase and chloramphenicol acetyltransferase synthesized in wheat germ extracts (7), and the DnaK and GroE systems were implicated in the initial folding of rhodanese in an *E. coli in vitro* translation system (8). The eukaryotic chaperones Hsp70, Hsp40, and TRiC assemble on nascent firefly luciferase and promote its folding (9). Recently, a role for ATP-dependent factors and PPIases in the folding of bacterial luciferase synthesized in reticulocyte lysates was reported (10). No evidence exists for association of PPIases with nascent polypeptide chains.

We describe the identification of trigger factor, a previously identified cytosolic *E. coli* protein implicated in translocation of pro-OmpA into inner membrane vesicles (11, 12), as a nascent chain-associated protein. Trigger factor has sequence homology to PPIases of the FK506-binding protein (FKBP) family and prolyl isomerase activity *in vitro.*

MATERIALS AND METHODS

Purification of Trigger Factor. Strain DH5α harboring plasmid pTIG2 encoding the *tig* gene under control of the p_{araB} promoter (13) was grown at 37°C in LB medium (Difco) containing 0.2% arabinose to an OD_{600} of 1.0. Cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris·HCl/6 mM $MgCl_2$/30 mM NH_4Cl/5 mM dithiothreitol, pH 7.5) and lysed in a French press. After removal of debris by centrifugation (30,000 × *g*, 30 min, 4°C), ribosomes were collected by centrifugation (150,000 × *g*, 4 hr, 4°C), resuspended in buffer A, and centrifuged through a 4-fold volume sucrose cushion (0.7 M sucrose in buffer A, 220,000 × *g*, 4 hr, 4°C). The sucrose cushion centrifugation was repeated in the presence of 0.3 M KOAc. These ribosomes were resuspended in buffer A/1.5 M KOAc and subjected to a third sucrose cushion centrifugation. The supernatant was dialyzed against buffer B [20 mM Tris·HCl/50 mM NaCl/5 mM dithiothreitol/5% (vol/vol) glycerol, pH 7.5], applied to an ion-exchange chromatography column (Protein Pak Q 8HR, Waters), and eluted by a gradient of 0.05–0.5 M NaCl in buffer B. Trigger factor eluted at 0.2 M NaCl (purity ≥ 95%) and was frozen in liquid N_2 and stored at −80°C. Protein concentrations were determined by using the Bio-Rad protein assay with lysozyme as standard.

Purification of Nontranslating Wheat Germ Ribosomes. Wheat germ extract (14) was incubated with 1 mM puromycin/0.5 M KOAc (20 min, 25°C) followed by sucrose cushion centrifugation {0.7 M sucrose in buffer C [25 mM Hepes–KOH/5 mM $Mg(OAc)_2$/5 mM dithiothreitol, pH 7.6], 0.5 M KOAc} as described above. Ribosomal pellets were resuspended in buffer C/50 mM KOAc to yield a ribosome concentration of 2 nmol/ml.

Translation of Preprolactin (PPL) in Wheat Germ Extract and Photocrosslinking to *E. coli* Proteins. Translation of PPL86 and PPL86mut in the presence of ε-4-(3-trifluoromethyldiazirino)benzoic acid (TDBA)-lysyl-tRNA (15) and [^{35}S]methionine and preparation of ribosome–nascent chain complexes (RNCs) were as described (16). *E. coli* extracts were prepared from strain BG87 (13) grown in the presence of arabinose (wild-type levels of trigger factor, TIG^+) or glucose (trigger factor-depleted cells, TIG^-) as detailed for the strain

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Abbreviations: PPIase, peptidyl-prolyl-cis/trans-isomerase; RNC, ribosome–nascent chain complex; PPL, preprolactin; TDBA, 4-(3-trifluoromethyldiazirino)benzoic acid; FKBP, FK506-binding protein.

overproducing the *E. coli* protein P48 (Ffh) (17). Four microliters of extract was added to 10 μl of RNCs in buffer C/50 mM KOAc and incubated on ice for 30 min. Samples were UV irradiated, precipitated with trichloroacetic acid, and subjected to SDS/PAGE. Crosslinking experiments were evaluated on a FUJIX BAS 1000 phosphorimager.

RESULTS

Puromycin-Insensitive Association of DnaK and GroEL with Ribosomes. Cell-free transcription/translation was carried out in a S-30 extract in the presence of [^{35}S]methionine with or without *lacZ* template. To stabilize or disrupt RNCs, translation reactions were stopped by addition of chloramphenicol or puromycin, respectively. Translation mixtures were salt treated to distinguish ionic and hydrophobic protein interactions, followed by separation of ribosomes and associated proteins from free protein by centrifugation through sucrose cushions. Ribosome-bound nascent β-galactosidase chains ranged in size from less than 20 kDa to full length (Fig. 1*A*). Puromycin treatment released 90% of the nascent chains.

To analyze the protein composition of the ribosomal fractions, the gel shown in Fig. 1*A* was silver stained. In addition to the ribosomal proteins, about 20 proteins were detected, many of which we identified (Fig. 1*B*). The chaperones DnaK and GroEL were present in the ribosomal fractions (Fig. 1 *B* and *C*). Under the conditions used, purified DnaK and GroEL proteins did not migrate through the sucrose cushion (not shown). DnaK amounts in the ribosomal fractions were unaffected by ongoing transcription/translation and high-salt treatment, whereas GroEL was displaced from nontranslating ribosomes by high salt. Both chaperones remained associated with ribosomes after puromycin-mediated release of nascent β-galactosidase. Addition of apyrase to deplete ATP, thus disfavoring substrate release from chaperones, did not affect the amount of the two chaperones associated with ribosomal fractions (not shown).

Trigger Factor Binds to Translating Ribosomes and Is Released by Puromycin. The only protein detected to specifically associate with translating ribosome fractions and to dissociate efficiently upon puromycin treatment was a protein of apparent molecular mass of 58 kDa (Fig. 1*B*, arrow). This behavior was more apparent after high-salt treatment. N-terminal sequencing of the 58-kDa protein (MQVSVET) identified it as trigger factor (13). In agreement, a polyclonal antibody raised against trigger factor specifically recognized the 58-kDa protein in an immunoblot (Fig. 1*C*). Thus, trigger factor associates with β-galactosidase-translating ribosomes and dissociates upon release of the nascent chains.

Trigger Factor Is Associated with Ribosome-Bound Nascent PPL. To obtain direct evidence for the association of trigger factor with nascent polypeptide chains, we used a crosslinking approach. Truncated mRNA was translated in a wheat germ extract, leading to accumulation of arrested nascent chains on the ribosomes. Translation reactions were carried out in the presence

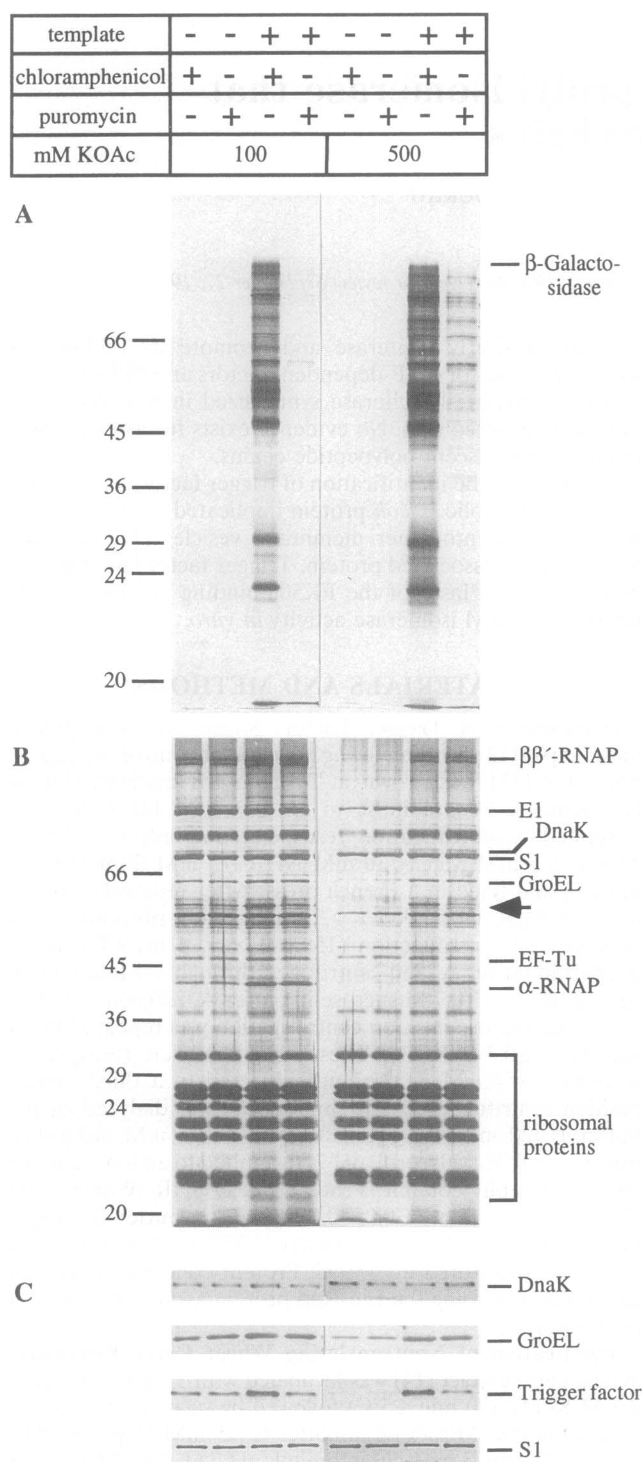

FIG. 1. Puromycin-sensitive association of trigger factor with ribosomes synthesizing β-galactosidase. A Zubay-type S-30 extract was prepared from strain MRE600 as described (18). Plasmid pML3A1 (19) carrying the *lacZ* gene with authentic ribosomal binding site under control of the A1 promoter of phage T7 served as template. Unsynchronized transcription/translation reactions of 105 μl total volume were carried out for 30 min at 37°C with 0.1 mCi of [^{35}S]methionine (Amersham; 1 mCi = 37 MBq) according to ref. 18. Chloramphenicol was added to 1 mM immediately before transfer of the tubes to an ice-water bath. KOAc and puromycin (Sigma) were added at 0°C to 0.1 or 0.5 M and 1 mM, respectively. After incubation for 15 min at 0°C, samples were subjected to centrifugation (5 min, 14,000 × *g*, 4°C), layered onto sucrose cushions (0.7 M sucrose in buffer C containing 0.1 or 0.5 M KOAc), and centrifuged at 250,000 × *g* for 30 min at 3°C. Ribosomal pellets were washed and resuspended in buffer C followed by quantification of ribosomal RNA (A_{260}). Two picomoles of ribosomal material was subjected to 12% acrylamide SDS/PAGE followed by silver staining and autoradiography of the gel or immunoblotting. (*A*) [^{35}S]Methionine-labeled nascent β-galactosidase chains; the position of full-length β-galactosidase is indicated. (*B*) The protein composition of the ribosomal fractions was analyzed by silver staining of the same gel shown in *A*. Proteins identified by their molecular masses (kDa) are the α, β, and β′ subunits of RNA polymerase (RNAP) and elongation factor EF-Tu. Proteins identified by N-terminal sequencing and/or immunoblotting are pyruvate dehydrogenase subunit E1, DnaK, ribosomal protein S1, GroEL, and trigger factor. The arrow indicates the 58-kDa protein released from translating ribosomes by puromycin treatment and identified as trigger factor. (*C*) Ribosomal fractions were probed with antibodies specific for DnaK, GroEL, trigger factor, and, as internal standard, ribosomal protein S1.

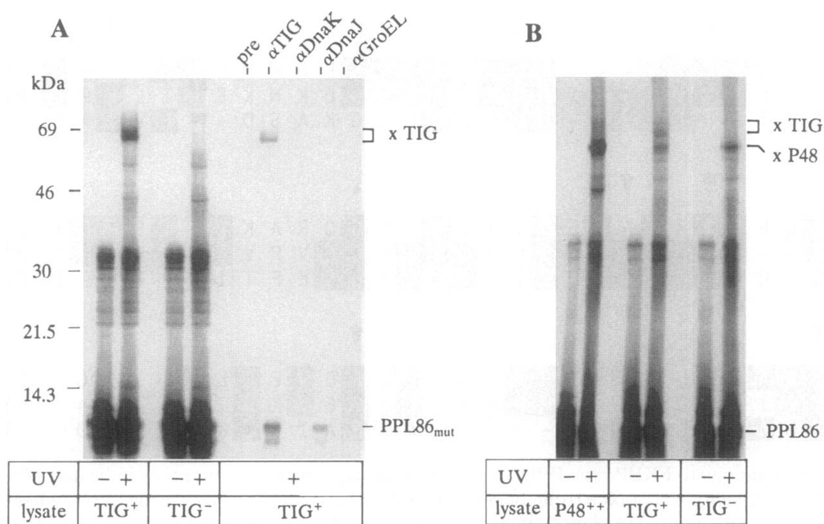

FIG. 2. Trigger factor is crosslinked to nascent PPL. (*A*) PPL86mut RNCs were incubated with *E. coli* lysates containing (TIG^+) or not containing (TIG^-) trigger factor. After crosslinking, aliquots were immunoprecipitated by preimmune serum and antisera against DnaK, DnaJ, GroEL, and trigger factor. All samples were analyzed by 9–15% acrylamide gradient SDS/PAGE followed by detection of [^{35}S]methionine-labeled protein by using phosphorimaging. × TIG indicates the position of the crosslinking product specific for trigger factor. (*B*) PPL86 RNCs were incubated with TIG^+ and TIG^- lysates and a lysate prepared from a P48-overproducing strain ($P48^{++}$), crosslinked, and analyzed as in *A*. × TIG and × P48 indicate the positions of crosslinking products specific for the particular protein.

of ε-TDBA-lysyl-tRNA, allowing UV-dependent crosslinking of nascent chains to associated proteins. The extremely short half-life and high unspecific reactivity of the carbene radical intermediate of TDBA allows crosslinking only if the ligand is in direct contact. To investigate whether trigger factor associates with both secretory and nonsecretory nascent chains, we used the N-terminal 86 amino acids of PPL (PPL86) and a nonsecretory PPL mutant (PPL86mut) (17) as model substrates. RNCs harboring either PPL86 or PPL86mut were isolated and incubated with *E. coli* extracts prepared from the conditional trigger factor depletion strain BG87 (13) containing (TIG^+) or depleted of (TIG^-) trigger factor.

Translation of the truncated mRNAs generated the expected 8-kDa products and, as observed earlier (16), several products of 20–35 kDa presumably resulting from mRNA heterogeneity (Fig. 2). In the case of PPL86mut, UV irradiation generated products of molecular masses between 44 and 66 kDa (Fig. 2*A*). The minor 44- and 55-kDa products were also obtained in the absence of *E. coli* lysate (not shown) and thus result from association of wheat germ proteins with PPL86mut. The major 66-kDa crosslinking product, appearing as a double band, was obtained only when the TIG^+ lysate was added. It was specifically immunoprecipitated with a trigger factor-specific antibody (Fig. 2*A*). In the case of PPL86, crosslinking generated *E. coli*-specific products of 60 and 66 kDa (Fig. 2*B*). Similar to PPL86mut, a 66-kDa trigger factor-PPL86 crosslinking product appeared, provided that TIG^+ lysate was added. In contrast to PPL86mut, a 60-kDa product appeared with both lysates. It resulted from crosslinking of the P48 component of the *E. coli* signal recognition particle to PPL86 as demonstrated previously (17). This was confirmed by use of a lysate from a P48-overproducing strain ($P48^{++}$) leading to increased amounts of the 60-kDa product (Fig. 2*B*) and immunoprecipitation with P48-specific antisera (not

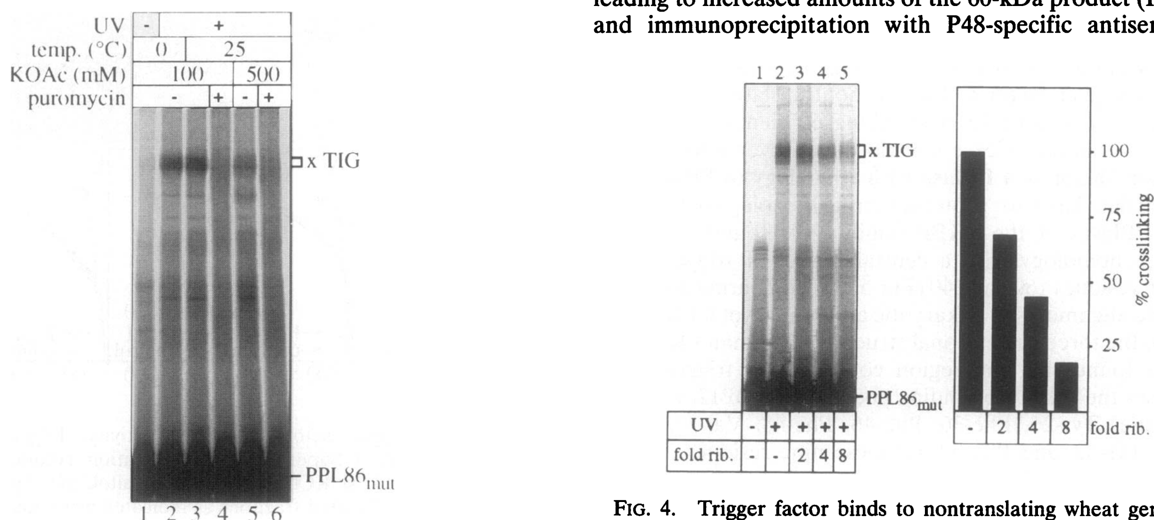

FIG. 3. Trigger factor is crosslinked to ribosome-bound but not to puromycin-released PPL. PPL86mut RNCs were incubated with equimolar amounts of purified trigger factor for 30 min on ice. An aliquot of this incubation was directly UV-irradiated as control, and the remainder was transferred to 25°C for 15 min for treatment with KOAc and puromycin (1 mM) as indicated, followed by UV irradiation and analysis as described in the legend to Fig. 2.

FIG. 4. Trigger factor binds to nontranslating wheat germ ribosomes. PPL86mut RNCs were incubated with equimolar amounts of purified trigger factor and an *x*-fold molar excess of nontranslating wheat germ ribosomes for 30 min on ice at 50 mM KOAc. The samples were UV irradiated and analyzed as described in the legend to Fig. 2. The trigger factor-specific crosslinking products (× TIG) were quantified and normalized to the total activity present in the individual lane by phosphorimager evaluation. The crosslinking efficiency in the absence of nontranslating wheat germ ribosomes was set as 100%.

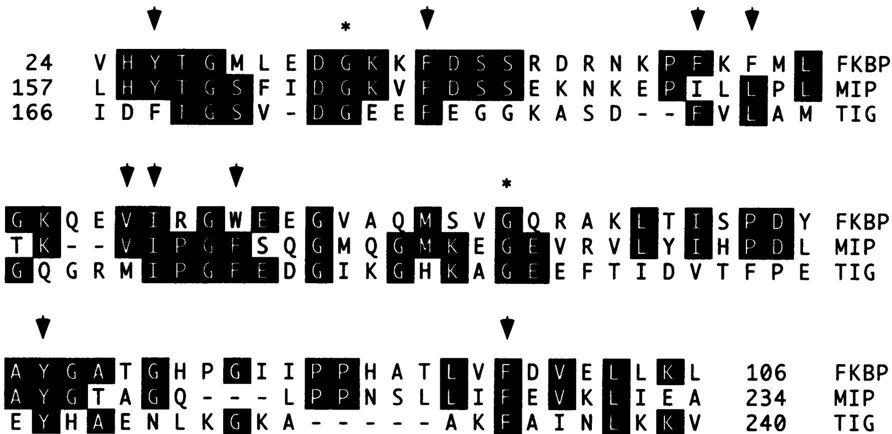

FIG. 5. Trigger factor shows homology to FKBP-type PPIases. Amino acid sequences of segments as indicated of human FKBP12cy, *Chlamydia trachomatis* MIP27, and *E. coli* trigger factor were taken from the Swiss-Prot data base and aligned by using the CLUSTAL method within the MEGALIGN software package. Black boxes indicate amino acid identity in at least two sequences. Arrows indicate amino acids forming the substrate-binding pocket of human FKBP12cy. Asterisks indicate the two glycine residues that are entirely conserved within the FKBP family.

shown). Together, trigger factor can be crosslinked to both types of arrested nascent chains, and we interpret this as association.

Trigger Factor Is Not Stably Associated with PPL86mut After Puromycin Release. We investigated whether trigger factor remains associated with nascent polypeptide chains after their release from ribosomes. PPL86mut RNCs were incubated with purified trigger factor. Then the nascent chains were released by addition of puromycin prior to crosslinking. The crosslinking efficiency of trigger factor to PPL86mut was strongly reduced when puromycin treatment preceded UV irradiation, at both low- and high-salt conditions (Fig. 3).

Trigger Factor Binds to Nontranslating Wheat Germ Ribosomes. Trigger factor was shown previously to copurify with *E. coli* ribosomes (20). The ability of trigger factor to bind to ribosomes might be a prerequisite for its efficient association with nascent polypeptides. We therefore tested whether trigger factor binds to nontranslating wheat germ ribosomes. In a competition experiment, increasing amounts of nontranslating ribosomes were incubated with PPL86mut RNCs and limiting amounts of trigger factor, followed by crosslinking. The yield of crosslinked product decreased with increasing concentration of non-translating ribosomes to less than 20% of the control without added ribosomes (Fig. 4). This indicates the ability of trigger factor to bind to nontranslating ribosomes. However, a reduction in crosslinking efficiency by 50% required a 4-fold molar excess of ribosomes over RNCs.

Trigger Factor Is a PPIase with Homology to FKBPs. We searched data bases for proteins sharing homology with trigger factor. PPIases of the FKBP family were found to exhibit sequence homology with a central region of trigger factor between residues 160 and 240 (Fig. 5). By comparing published sequence alignments of eukaryotic and prokaryotic FKBPs (4, 21) with the three-dimensional structure of human FKBP12cy (22) we found that the region conserved in trigger factor comprises the substrate-binding pocket of FKBP12cy formed by residues Tyr-26, Phe-36, Phe-46, Phe-48, Val-55, Ile-56, Trp-59, Tyr-82, and Phe-99. Of these nine residues, five are identical in trigger factor (Phe-177, Phe-185, Ile-195, Tyr-221, and Phe-233), two are conservative exchanges (Phe-168 and Phe-198), and two are nonconservative exchanges (Leu-187 and Met-194). Furthermore, two entirely conserved glycine "signature" residues (Gly-34 and Gly-70) (21) exist in trigger factor as well (Gly-174 and Gly-208). The homology of trigger factor with the *C. trachomatis* MIP27 member of the FKBP family is even more striking. Both proteins have a phenylalanine in place of the tryptophan at the corresponding position 59 of FKBP12cy. For FKBP12cy, this residue was implicated in contacting the inhibitor FK506 and substrate proline residues.

We tested whether trigger factor has PPIase activity *in vitro*, using a photometric standard assay (23). This assay detects PPIase activity by measuring the efficiency of the *trans*-isomer-specific proteolytic cleavage of the substrate succinyl-Ala-Phe-Pro-Phe-4-nitroanilide containing a mixture of *cis* and *trans* Phe-Pro bonds. Our trigger factor preparation catalyzed the *cis–trans* isomerization of the Phe-Pro peptide bond (Fig. 6). To further correlate PPIase activity with trigger factor, a gel filtration fractionation of this preparation was carried out. Here, PPIase activities of individual fractions correlated with the amount of trigger factor present (Fig. 6). In addition, the PPIase activity could be immunodepleted by trigger factor-specific antibodies (not shown). We conclude that trigger factor is a PPIase. To determine rate constants of the uncatalyzed and catalyzed reactions, trigger factor at a final concentration of 200 nM was added 40 sec after the addition of

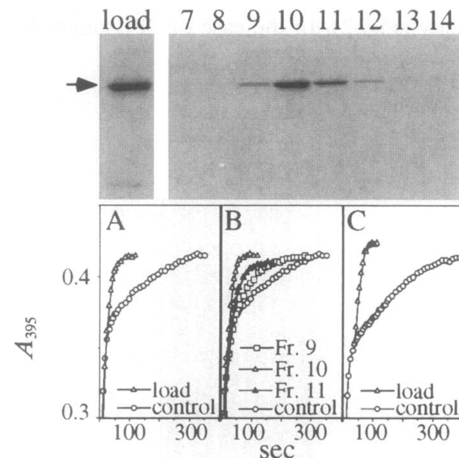

FIG. 6. Trigger factor has PPIase activity. Trigger factor was fractionated on a Superdex 75 gel filtration column in 35 mM Hepes–KOH/0.1 M KCl/1 mM dithiothreitol, pH 7.6. Aliquots of column load and eluted fractions as indicated were analyzed by 12% acrylamide SDS/PAGE and silver staining (*Upper*). PPIase activities of load (*A*) and identical volumes of trigger factor peak fractions (*B*) were determined at 10°C by using a Varian DMS 200 spectrophotometer and succinyl-Ala-Phe-Pro-Phe-4-nitroanilide (Bachem) as substrate as described (23) (*Lower*). Trigger factor of load and peak fraction 10 were tested at a final concentration of 200 nM. For determination of rate constants, trigger factor (200 nM) was added 40 sec after addition of substrate (*C*).

substrate peptide to the protease. First-order rate constants of the uncatalyzed and catalyzed reactions were 4.2×10^{-3} s^{-1} and 4.9×10^{-2} s^{-1}, respectively.

DISCUSSION

The key results of this study are that trigger factor is a major nascent polypeptide chain-associated protein of *E. coli* and that it exhibits PPIase activity *in vitro*. Because trigger factor also binds to ribosomes (this study; ref. 20), we propose that *E. coli* harbors a PPIase on its ribosomes which acts cotranslationally to catalyze the folding of newly synthesized proteins.

Our search for proteins associated with ribosome-bound nascent β-galactosidase also identified the DnaK and GroEL chaperones in ribosomal fractions. This association, however, was independent of the presence of nascent chains. DnaK and GroEL might have roles in the assembly and/or maintenance of the transcription/translation machinery which involve association with ribosomes. This assumption is consistent with the finding that missense mutations in *dnaK* result in ribosome assembly defects (24). Association of DnaK and GroEL with ribosomes as part of such roles might have prevented detection of additional interactions of these proteins with nascent chains.

In contrast to these chaperones, trigger factor dissociated efficiently from ribosomes after puromycin treatment. As demonstrated by crosslinking, trigger factor is associated with arrested nascent PPL chains. On the basis of the N-terminal location of the ε-TDBA-lysine residues at positions 4 and 9 in PPL, trigger factor can associate with the extreme N termini of secretory nascent chains. It shares this ability with the P48 component of the *E. coli* signal recognition particle (17). In contrast to P48, trigger factor also associated with a nonsecretory mutant of PPL, where it was the only crosslinked *E. coli* protein. Thus, trigger factor can associate with nascent polypeptide chains irrespective of their cellular destination.

On the basis of our results and published work (20) we propose that trigger factor binds to a site on translating ribosomes that is in proximity to the exit site of nascent chains and allows efficient interaction of trigger factor with the polypeptide chain. Three lines of evidence support this proposal. First, trigger factor copurifies with *E. coli* ribosomes and rebinds to the large ribosomal subunit (20) containing the exit site for nascent polypeptides. Trigger factor also binds to wheat germ ribosomes, suggesting conservation in evolution of the trigger factor binding site and validating the use of the heterologous translation system for studying nascent chain association. Second, only *E. coli* ribosomes harboring nascent chains bind trigger factor in a salt-resistant fashion. We infer that either the nascent chain itself or the tightly coupled state of the translating ribosome strengthens the binding of trigger factor to RNCs. The puromycin-mediated release of trigger factor from translating ribosomes is compatible with both possibilities. Third, the ribosome-bound state of the nascent polypeptide chain is required for efficient association with trigger factor. This is indicated by the low efficiency of trigger factor crosslinking to nascent PPL after its release from ribosomes. Also, we failed to co-immunoprecipitate trigger factor with β-galactosidase puromycyl fragments (not shown). It is possible that association of trigger factor requires a rather unfolded conformation of the substrate. Consistent with this possibility is that trigger factor was reported to functionally interact with pro-OmpA only when this substrate was unfolded by urea (12).

A key to understanding the role of trigger factor in protein biogenesis is the demonstration of its PPIase activity. In an independent study, trigger factor was identified as a ribosome-associated PPIase that can catalyze protein folding *in vitro* (25). Taking these results together, we propose that one important biological activity of trigger factor is to be a folding catalyst acting cotranslationally. Interestingly, depletion of trigger factor does not significantly perturb growth of *E. coli* cells (13). The existence of at least six different PPIase activities in *E. coli* (G. Fischer, personal communication) might constitute a back-up system which complements lack of trigger factor activity in depleted cells. Clearly, identification of the physiological role of trigger factor will require dissection of the functional network of cytosolic PPIases of *E. coli*.

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