

The Amino-terminal 118 Amino Acids of *Escherichia coli* Trigger Factor Constitute a Domain That Is Necessary and Sufficient for Binding to Ribosomes*

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***Escherichia coli* trigger factor has prolyl-isomerase and chaperone activities and associates with nascent polypeptide chains. Trigger factor has a binding site on ribosomes, which is a prerequisite for its efficient association with nascent chains and its proposed function as a cotranslational folding catalyst. We set out to identify the domain of trigger factor that mediates ribosome binding. Of a series of recombinant fragments, the amino-terminal fragments, TF (1–144) and TF (1–247), cofractionated with ribosomes from cell extracts and re-bound to isolated ribosomes *in vitro*. They competed efficiently with full-length trigger factor for stoichiometric binding to a single site on the large ribosomal subunit. However, TF (1–144) and TF (1–247) differed from full-length trigger factor in that their association with ribosomes was not strengthened by the presence of nascent chains, indicating a role for carboxyl-terminal trigger factor segment in sensing the translational status. The domain responsible for ribosome binding was further investigated by limited proteolysis of recombinant fragments. A stable domain comprising the amino-terminal 118 residues was identified that was still capable of ribosome binding and thus represents a novel structural and functional element of trigger factor.**

Trigger factor was first identified in *Escherichia coli* (1) but meanwhile has also been found in other bacteria (2). It was isolated by its activity to promote *in vitro* the translocation of precursors of the outer membrane protein A (proOmpA) into membrane vesicles (3, 4). Trigger factor is associated with ribosomes prepared from cell extracts and binds to purified large ribosomal subunits (5) known to contain the exit site for nascent polypeptide chains. These findings led to the earlier proposal that trigger factor acts as a secretion-specific chaperone to shuttle precursors of secretory proteins from their place of synthesis to membrane translocation sites (5). However, *E. coli* cells depleted of trigger factor do not show secretion defects (6).

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Recently, trigger factor was identified as a ribosome-associated peptidyl-prolyl-*cis/trans*-isomerase (PPIase)¹ (7). The purified protein catalyzed the prolyl isomerization-dependent refolding of the unfolded protein substrate RNaseT1 much more efficiently than other PPIases tested before (7). This high efficiency is due to the cooperation of PPIase and chaperone activities within the protein (8). In independent studies, using *in vitro* translation systems, trigger factor was identified as a major cross-linking partner for nascent polypeptide chains of cytoplasmic and secretory proteins (9, 10). The association of trigger factor with ribosomes was found to be sensitive to the translational status. Translating ribosomes formed complexes with trigger factor that were resistant to high salt treatment and disrupted by puromycin-mediated release of the nascent polypeptide chains, whereas nontranslating ribosomes formed salt-sensitive complexes only (10). Together, a scenario was proposed in which trigger factor, by virtue of its PPIase activity and an additional chaperone-like function, assists the folding of nascent polypeptide chains as they emerge from the ribosome (7, 9, 10). In addition, trigger factor functionally cooperates with the GroEL chaperone, as shown by its activity to promote the GroEL-dependent degradation of polypeptides *in vivo* (11).

The PPIase activity was localized in a central domain of trigger factor between amino acids 145 and 247/251 (12, 13). This assignment was predicted on the basis of a sequence similarity to FK506 binding protein (FKBP)-type PPIases (10, 14) and a hydrophobic cluster analysis (14) and experimentally verified by limited proteolysis of the native protein (12, 13). The isolated PPIase domain was ~1000-fold less active than full-length trigger factor in refolding of RNaseT1, indicating that the flanking amino- and/or carboxyl-terminal parts of its polypeptide chain are required for high refolding activity (8).

We now set out to identify the domain of trigger factor that mediates ribosome binding. This domain is likely to be central to a putative mechanism that targets trigger factor to nascent polypeptide chains. Assuming a modular structure of the protein, we designed recombinant fragments of trigger factor on the basis of the structural information obtained by limited proteolysis. They comprise the amino-terminal part of the polypeptide chain, TF (1–144), the central FKBP domain, TF (145–247), the carboxyl-terminal portion, TF (248–432), or combinations thereof, TF (1–247) and TF (145–432). We found that the amino-terminal 118 amino acids of trigger factor as part of TF (1–144) are necessary and sufficient for specific ribosome binding.

¹ The abbreviations used are: PPIase, peptidyl-prolyl-*cis/trans*-isomerase; FKBP, FK506 binding protein; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Generation of Carboxyl-terminally His-tagged Trigger Factor Fragments—Plasmid pTIG2 (6) containing the full-length, wild-type *tig* gene from *E. coli* served as a template for the polymerase chain reaction-based amplification of different gene fragments. The gene fragment corresponding to the first 144 codons of *tig* was amplified using the primer pair 5'-GGC CGG ATC CAT GCA AGT TTC AGT TGA AA-3' (P1) and 5'-GGC CGG ATC CCA GAG TAT CCA GCA TGC CG-3' (P4), the fragment corresponding to codons 145–247 using the primer pair 5'-GGC CGG ATC CCG TAA ACA GCA GGC GAC CT-3' (P2) and 5'-GGC CGG ATC CTT CCG GCA GTT CAC GCT CT-3' (P5), and the fragment corresponding to codons 248–432 using the primer pair 5'-GGC CGG ATC CCT GAC TGC AGA ATT CAT CA-3' (P3) and 5'-GGC CGG ATC CCG CCT GCT GGT TCA TCA GC-3' (P6). Fragments corresponding to codons 1–247 and 145–432 were amplified with the primer pairs P1/P5 and P2/P6, respectively. A gene fragment encoding the amino-terminal 118 amino acids of trigger factor was polymerase chain reaction-amplified using the primers P1 and 5'-GGC CGG ATC CCT CGA GTT CAA CTT CCG GA-3' (P7). Polymerase chain reactions were carried out with Pwo DNA polymerase (Boehringer Mannheim) according to the manufacturer's instructions. Amplification products were purified, digested with *Bam*HI, and ligated into the single *Bam*HI site of the expression vector pDS56 RBSII, 6 × His (15). The resulting expression products have four additional amino acids at the amino terminus (MRGS) and eight additional amino acids at the carboxyl terminus (RSHHHHHH). Ligations were transformed into *E. coli* DH5 α containing pDMI,1 which encodes *lacI^q* (16) followed by sequencing of the inserts. Clones with wild-type *tig* sequences were used for expression and purification of trigger factor fragments. A construct for the expression of full-length, His-tagged trigger factor was obtained by ligation of the two overlapping polymerase chain reaction-derived gene fragments at the single *Acc*I restriction site.

For purification of the proteins, expression of the trigger factor fragments in DH5 α containing pDMI,1 was induced at an A_{600} of 0.5 with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h at 30 °C in LB medium. Cells were harvested by centrifugation in the cold and resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme, and 15% (w/v) sucrose, pH 7.6 at 4 °C). Following incubation at 4 °C for 15 min, the cells were frozen at –80 °C, thawed slowly on ice, and lysed by the addition of a 4-fold volume of cold distilled water and brief sonication. After centrifugation (30,000 × *g* for 30 min at 4 °C), the cleared supernatants were loaded onto Ni²⁺-NTA agarose (Qiagen) columns equilibrated in buffer A (20 mM Tris-HCl, 2 mM 2-mercaptoethanol, pH 7.6 at 4 °C) and 100 mM NaCl. Columns were washed with three column volumes buffer A containing 100, 200, and 500 mM NaCl, followed by step elution with 1 column volume each of 10, 20, 30, 40, 50, 100, and 200 mM imidazole in buffer A and 100 mM NaCl. Trigger factor peak fractions were either pooled and dialyzed against trigger factor storage buffer (20 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, and 2 mM 2-mercaptoethanol) or 4-fold diluted with distilled water and rechromatographed on a Protein Pak Q 8 HR (Waters) strong anion exchange column, essentially as described before, for native full-length trigger factor (12). Purity of the individual fragments was assessed by SDS-PAGE analysis and Coomassie Brilliant Blue staining. Protein concentrations were determined using the BCA-kit (Pierce) and bovine serum albumin as a standard.

Isolation of Ribosome-Trigger Factor Complexes *ex Vivo*—DH5 α derivatives expressing the different trigger factor fragments were cultured in LB medium at 30 °C to an A_{600} of 0.5. Low level expression of trigger factor fragments was achieved by the addition of isopropyl-1-thio- β -D-galactopyranoside to 20 μ M for 1 h. 20-ml aliquots of the cultures were then rapidly cooled to 0 °C in an ice-water bath and centrifuged at 4 °C to harvest the cells. Cells were lysed essentially as described above except that the Mg²⁺ concentration was permanently kept at 10 mM. Crude lysates were cleared by centrifugation (15,000 × *g* for 15 min at 4 °C) and layered onto sucrose cushions (20% (w/v) sucrose in 20 mM Tris-HCl, 100 or 500 mM KCl, 10 mM MgCl₂, and 5 mM 2-mercaptoethanol, pH 7.6 at 4 °C; three volumes cushion per volume of lysate). Ribosomes were pelleted at 213,000 × *g* for 1 h at 4 °C in a TLA-100 rotor (Beckman). Ribosomal pellets were resuspended in SDS-PAGE sample buffer, and aliquots of ribosomes and postribosomal supernatant (50% of the corresponding amount of ribosomal pellets) were analyzed on 13.5% acrylamide denaturing gels. Gels were blotted onto nitrocellulose, which was then probed with a trigger factor-specific antiserum from rabbit and a chemiluminescence-based detection system (BM blotting substrate, Boehringer Mannheim).

Rebinding of Trigger Factor to Ribosomes—Ribosomes were purified

from a derivative of the *E. coli* C600 strain. Briefly, an inducer-regulated chromosomal *tig* allele was introduced from strain BG87 (6) into C600 by P1vir transduction and selection of Amp^r transductants. Transductants show arabinose-dependent expression of the chromosomal *tig* gene. To prepare ribosomes depleted of endogenous trigger factor, C600 transductants were grown in the absence of arabinose. Ribosomes were purified as described before (10), omitting, however, the high salt wash step, and stored in ribosome buffer (10 mM Tris-HCl, 6 mM MgCl₂, 60 mM NH₄Cl, and 4 mM 2-mercaptoethanol, pH 7.6) at –80 °C. Analytical sucrose gradient centrifugation showed a monosome:subunit ratio of 70:30%. Trigger factor fragments were added at the indicated concentrations followed by 60-min incubation at 30 °C. Samples were then layered onto a 3-fold volume of 20% (w/v) sucrose in buffer A (100 mM KCl, see above). Following high speed centrifugation as described in the previous section, supernatants and pellets were separated and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. For sucrose gradient centrifugation, four A_{260} units of preformed ribosome-TF (1–144) complexes were loaded onto 12 ml of 10–30% (w/v) linear sucrose gradients in buffer B (20 mM Tris-HCl, 100 mM NH₄Cl, and 5 mM 2-mercaptoethanol, pH 7.6) containing 1 mM MgCl₂ (subunit dissociation conditions) or 6 mM MgCl₂ (subunit association conditions). Gradients were run in a Beckman SW40 Ti rotor at 36,000 rpm for 100 min at 3 °C followed by fractionation from the tube bottom (40 fractions of 300 μ l each). SDS-PAGE analysis was done on 13.5% (w/v) acrylamide denaturing gels, followed by blotting onto nitrocellulose filters. Filters were probed with antisera raised against trigger factor or ribosomal proteins S7 and L7/L12 and detected as before.

Limited Proteolysis of Trigger Factor Fragments—For analysis by SDS-PAGE, 40 μ g of trigger factor fragments were incubated with 80 ng of proteinase K (Boehringer Mannheim) in a final volume of 200 μ l of 10 mM Tris-HCl (pH 8), 1 mM CaCl₂ at 30 °C. At the time points indicated, aliquots were taken, mixed with phenylmethylsulfonyl fluoride (final concentration, 1 mM) and analyzed by SDS-PAGE (13.5% (w/v) acrylamide) followed by silver staining. For amino-terminal sequencing, digested protein was separated by SDS-PAGE, blotted onto polyvinylidene difluoride membranes (Millipore) and microsequenced on a 473A Sequencer (Applied Biosystems). For mass spectroscopy, 100 μ g of TF (1–144) were digested with proteinase K for 20 min at 30 °C, mixed with phenylmethylsulfonyl fluoride and passed over a protein C₁₈ reversed phase high-performance liquid chromatography column (Vydac). Mass spectroscopy was carried out on a Kratos Compact MALDI 3 version 4.0. For ribosome binding studies, TF (1–247) at a final concentration of 30 μ M was digested with 6 nM proteinase K for 20 min at 30 °C, followed by the addition of phenylmethylsulfonyl fluoride to a 1 mM final concentration.

Miscellaneous—PPIase activity assays were carried out at 10 °C using the substrate Suc-Ala-Phe-Pro-Phe-pNA essentially as described (10). The concentration of trigger factor fragments in the assay was 50 nM. For gel filtration analyses, volumes of 200 μ l of the recombinant fragments (final concentration, ~20 μ M) were loaded on a Superdex 75 column (10/30, Pharmacia Biotech Inc.) and eluted at a flow rate of 0.5 ml/min in buffer A, 100 mM NaCl. The column was calibrated with bovine serum albumin (67 kDa), ovalbumin (45 kDa), and lysozyme (14 kDa) as standards and blue dextran 2000 to detect the void volume. *In vitro* transcription/translation reactions of the *lacZ* gene were carried out as before (10).

RESULTS

Purification of His-tagged Trigger Factor Fragments—The design of trigger factor fragments is based on previous results obtained by limited proteolysis of the full-length protein. Proteolysis by proteinase K, endoproteinase Glu-C (V8), and subtilisin generated stable fragments of ~12 kDa (amino acids 145–247/251) displaying full PPIase activity (12, 13). Based on this information, we prepared recombinant trigger factor fragments comprising the amino-terminal segment (TF 1–144), the central PPIase domain (TF 145–247), the carboxyl-terminal segment (TF 248–432), the overlapping fragments TF (1–247) and TF (145–432), and full-length trigger factor, TF (1–432) (Fig. 1). Each of these proteins carries at its carboxyl terminus a His-tag to facilitate purification. High level expression of the individual fragments in DH5 α host cells did not perturb cell growth and resulted in predominantly soluble recombinant fragments. Proteins were purified by affinity chromatography on Ni²⁺-NTA agarose and, depending on the purity achieved,

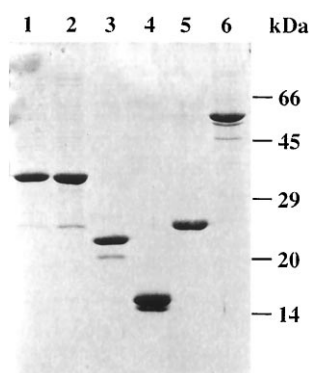


FIG. 1. Purified recombinant trigger factor fragments. The purified proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, TF (1–247); lane 2, TF (145–432); lane 3, TF (1–144); lane 4, TF (145–247); lane 5, TF (248–432); lane 6, TF (1–432).

a second chromatography step on a strong anion exchange column (Fig. 1).

To analyze the structural integrity of the fragments, PPIase activity assays, gel filtration analyses, and limited proteolysis were carried out. All fragments possessing the central FKBP domain displayed PPIase activity toward the oligopeptide substrate Suc-Ala-Phe-Pro-Phe-pNA. The specific activities were in the same range as reported before by us and others (7, 12, 13) for wild-type trigger factor and the isolated FKBP domain (Table I). Gel filtration analyses on a Superdex 75 column (linear separation range for globular proteins between 3 and 70 kDa) was carried out to further exclude that the purified fragments were misfolded and aggregated. For all fragments, 70% or more of the protein eluted within the included volume of the gel filtration column corresponding to monomeric or potentially dimeric states (Table I). Fragments containing the carboxyl-terminal domain, TF (1–432), TF (145–432), and TF (248–432), showed a tendency to aggregate at protein concentrations above $\sim 10 \mu\text{M}$, as indicated by a partial elution of these fragments in the void volume of the gel filtration column. Consistent with a previous report (13), the isolated PPIase domain eluted in a single sharp peak corresponding to a molecular mass 27 kDa. The result of limited proteolysis by proteinase K of full-length His-tagged trigger factor was indistinguishable from that observed before for untagged trigger factor (12). The central FKBP domain was entirely resistant to proteolysis in all fragments (data not shown; Fig. 7). The carboxyl-terminal domain of trigger factor, both as the isolated fragment TF (248–432) or as part of TF (1–432) and TF (145–432), was rapidly degraded without populating stable degradation intermediates (data not shown), as observed before for the untagged full-length protein (12, 13). The results of limited proteolysis of TF (1–144) and TF (1–247) are fully compatible with native polypeptide conformations and are detailed below (Fig. 7). Taken together, the purified recombinant trigger factor fragments, by three different criteria, display native or native-like conformations, allowing functional analyses. Caution may be required when interpreting results for the isolated carboxyl-terminal fragment, TF (248–432).

TF (1–247) and TF (1–144) Copurify with Ribosomes Prepared from Cell Extracts—To identify the domain of trigger factor that mediates ribosome binding, we first investigated a potential cosedimentation of the recombinant trigger factor fragments with ribosomal particles prepared from cell extracts. The positive binding controls were ribosomes prepared from DH5 α cells, which chromosomally encode untagged full-length trigger factor. At 100 mM KCl, 30–40% of the cellular trigger factor pool cofractionated with ribosomes (Fig. 2). An increase of the ionic strength to 500 mM during the centrifugation

TABLE I
Specific PPIase activities and gel filtration behavior of recombinant trigger factor fragments

Fragment ^a	PPIase activity K_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	M_r Sequence derived	M_r Gel filtration
TF (1–432)	0.69 ± 0.03	49,524.6	66,000 ^b
TF (1–144)	No catalysis	17,320.4	37,000
TF (145–247)	1.07 ± 0.004	12,931.1	27,000
TF (248–432)	No catalysis	22,303.3	39,000 ^b
TF (1–247)	0.72 ± 0.1	28,763.4	45,000
TF (145–432)	0.93 ± 0.07	33,719.3	52,000 ^b

^a All fragments carry a carboxyl-terminal His-tag.

^b Fractions ($\leq 30\%$) of these proteins eluted with the void volume ($M_r \geq 70,000$).

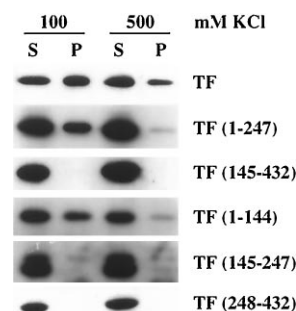


FIG. 2. Copurification of trigger factor fragments with ribosomes from cell extracts. *E. coli* DH5 α cells expressing the individual trigger factor fragments as indicated were fractionated into ribosomal pellets and postribosomal supernatants at KCl concentrations of 100 and 500 mM. Aliquots of supernatants (S) and ribosomal pellets (P) were applied to SDS-PAGE, followed by detection of trigger factor fragments by immunoblotting using a specific antiserum. Note that only 50% of the supernatant fractions were loaded.

through the dense sucrose cushion reduced the amount of cofractionating trigger factor 2–3-fold. Of the two larger fragments, TF (1–247) but not TF (145–432) cosedimented with ribosomes. Consistent with the failure of TF (145–432) to bind to ribosomal particles, neither of its subfragments TF (145–247) and TF (248–432) showed a ribosomal localization. By contrast, TF (1–144), the amino-terminal domain of trigger factor, cofractionated with ribosomes at 100 mM KCl, indicating that this fragment is necessary and sufficient for binding of trigger factor *in vivo*. However, binding of TF (1–247) and TF (1–144) to ribosomal particles was more susceptible to salt-induced dissociation than binding of the full-length protein.

TF (1–247) and TF (1–144) Rebind to Purified Ribosomes *In Vitro*—The ribosome-binding properties of the recombinant trigger factor fragments were further analyzed *in vitro* with purified ribosomes. To remove the endogenous trigger factor, ribosomes were prepared from trigger factor-depleted cells. This approach avoided high-salt washing of the ribosomes, which may cause microheterogeneity of the ribosomal particles. The ribosomal preparation used was mainly composed of 70S monosomes, as revealed by analytical sucrose gradient centrifugation at 6 mM Mg^{2+} (data not shown). Ribosomes were then incubated to equilibrium with a molar excess of trigger factor fragments and reisolated by centrifugation through sucrose cushions. Analysis of the ribosomal pellets and postribosomal supernatants by SDS-PAGE confirmed the previous observation that TF (1–247) and TF (1–144), but not TF (145–432), can bind to ribosomes (Fig. 3). Neither TF (145–247) nor TF (248–432) showed ribosome binding *in vitro* (data not shown). TF (1–144) is thus the smallest of the trigger factor fragments tested to bind to ribosomes *in vivo* and *in vitro*. Judged from the staining intensities (Fig. 3), binding of TF (1–247) and TF (1–144) to ribosomal particles shows an apparent saturation at a 1:1 stoichiometry indicative of a single ribosomal attachment site.

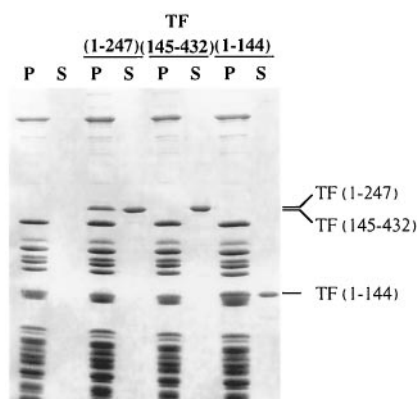


FIG. 3. TF (1-144) and TF (1-247) bind to ribosomes *in vitro*. Trigger factor fragments at $\sim 3 \mu\text{M}$ final concentration were incubated with trigger factor-depleted ribosomes ($2 \mu\text{M}$) to equilibrium, followed by sucrose cushion centrifugation to re-isolate the ribosomal particles. Identical aliquots of ribosomal pellets (*P*) and postribosomal supernatants (*S*) were separated by SDS-PAGE prior to staining of proteins with Coomassie Brilliant Blue.

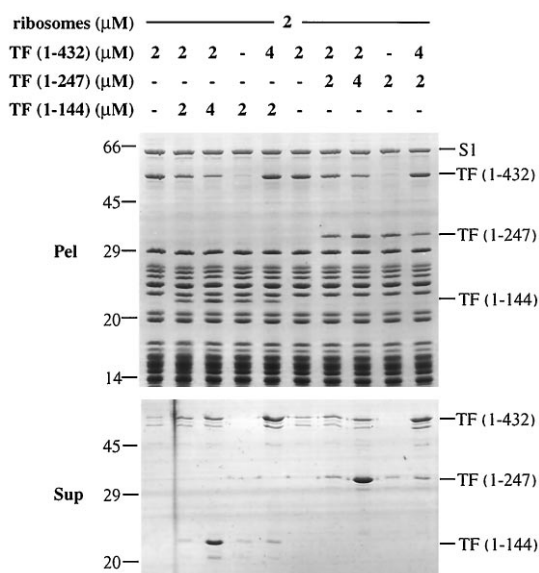


FIG. 4. Trigger factor, TF (1-144) and TF (1-247), compete for binding to a single ribosomal site. Ribosomes, trigger factor, and the fragments were coincubated to equilibrium at the concentrations indicated and subjected to sucrose cushion centrifugation at 100 mM salt. Identical aliquots of ribosomal pellets (*Pel*) and postribosomal supernatants (*Sup*) were applied to SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Trigger Factor and Its Subfragments TF (1-247) and TF (1-144) Compete for Binding to a Single Ribosomal Site—To substantiate the observation that ribosome binding by trigger factor is a function of its amino-terminal domain, direct competition experiments were carried out. Ribosomes were incubated to equilibrium with equimolar amounts of TF (1-432), TF (1-247), and TF (1-144), re-isolated by centrifugation, and analyzed by SDS-PAGE (Fig. 4). In agreement with the experiment shown in Fig. 3, visual inspection of the gel reveals that binding of the individual trigger factor fragments to ribosomes occurs at an apparent 1:1 stoichiometry. Co-incubation of equimolar, saturating amounts of TF (1-432) and TF (1-144) or TF (1-432) and TF (1-247) with ribosomes resulted in a $\sim 50\%$ reduction in binding of the individual fragments and a concurrent appearance of the proteins in the supernatant fractions. Moreover, a 2-fold molar excess of trigger factor or trigger factor fragments during the incubation with ribosomes

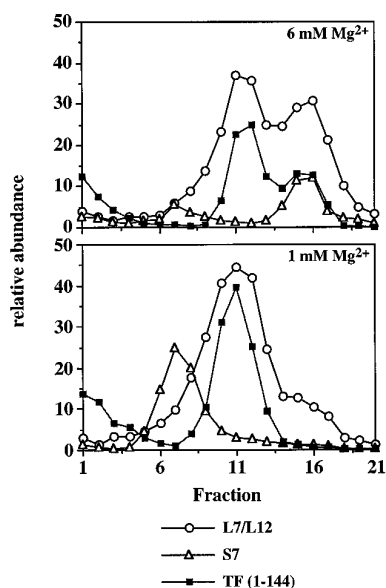


FIG. 5. TF (1-144) binds to the large ribosomal subunit. Preformed TF (1-144)-ribosome complexes were loaded onto 10–30% (w/v) sucrose gradients in buffer containing 6 mM Mg^{2+} or 1 mM Mg^{2+} to stabilize or destabilize, respectively, the coupled state of the ribosome. Gradients were run and fractionated as detailed under “Experimental Procedures,” followed by immunoblot analysis of gradient fractions with antisera specific for trigger factor, ribosomal protein S7 (to detect the small ribosomal subunit), and L7/L12 (to detect the large ribosomal subunit). Immunoblots were analyzed by laser scanning densitometry. Fraction 1 is the top of the gradient.

resulted in an equivalent binding to the ribosomal particles and appearance of unbound protein in supernatant fractions (Fig. 4). These results indicate that TF (1-432), TF (1-144), and TF (1-247) have the same binding specificity and apparent affinity toward a single ribosomal attachment site.

TF (1-144) Binds to the Large Ribosomal Subunit—The ribosomal binding site of trigger factor was shown earlier to map to the large ribosomal subunit (5). Direct competition of TF (1-144) and TF (1-432) for ribosome binding suggests that the amino-terminal fragment is capable of discriminating the ribosomal subunits. This was investigated by sucrose gradient centrifugation. We envisioned that free TF (1-144) may smear into the 30S area of the sucrose gradients, thereby mimicking binding to the small ribosomal subunit. To avoid this potential difficulty, we used as the starting material for the gradient centrifugations the ribosome-TF (1-144)-complex isolated by sucrose cushion centrifugation in the presence of high magnesium concentration. Four A_{260} units of this complex were centrifuged in sucrose gradients containing either high or low magnesium concentrations to stabilize or destabilize, respectively, the coupled state of the ribosome. Fig. 5 shows the distributions of TF (1-144), ribosomal S7 (to identify the small ribosomal subunit), and ribosomal L7/L12 (to identify the large ribosomal subunit) in the gradient fractions. Accordingly, TF (1-144) binds to 70S couples (*upper panel*, fractions 15 and 16) and 50S large ribosomal subunits (*upper panel*, fractions 11 and 12; *lower panel*, fractions 10–12). There was no binding to the small ribosomal subunit under both magnesium conditions (*both panels*, fractions 7 and 8). Thus, TF (1-144) further resembles the full-length protein in that it is capable of discriminating the ribosomal subunits and binding to large subunits only.

*TF (1-144) and TF (1-247) Are Incapable of Forming Salt-resistant and Puromycin-sensitive Complexes with Translating Ribosomes *in Vitro**—In an earlier study, we found that the association of trigger factor with ribosomes becomes salt-resis-

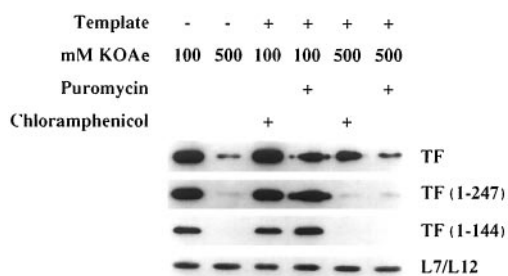


FIG. 6. Association of TF (1-144) and TF (1-247) with translating ribosomes *in vitro*. Transcription/translation reactions of *lacZ* were carried out in S-30 extracts at 37 °C in presence of [³⁵S]methionine to label newly synthesized β -galactosidase. Where indicated, template was omitted as a nontranslational control. Reactions were carried out in the absence of exogenously added trigger factor fragments (extract endogenous trigger factor only) or individual presence of TF (1-144) and TF (1-247) at 1 μ M final concentration. Transcription/translation was stopped by placing the tubes into an ice-water bath, followed by the addition of puromycin or chloramphenicol (1 mM final concentrations) and KOAc, as indicated. Samples were cleared by spinning at 15,000 \times g for 5 min at 4 °C and layered onto sucrose cushions containing 100 or 500 mM KOAc, respectively. Following high speed centrifugation, post-ribosomal supernatants were aspirated and discarded. Ribosomal pellets were analyzed by SDS-PAGE and immunoblotting with a trigger factor-specific antiserum. Puromycin reactions occurred with ~80% efficiency as revealed by liquid scintillation counting of ribosomal fractions. The addition of TF (1-144) or TF (1-247) did not perturb transcriptional or translational efficiency. As a loading control, immunoblots were re-probed with an antiserum specific for ribosomal L7/L12.

tant when translation occurs (10). These complexes were disrupted by puromycin-mediated release of the nascent polypeptide chains (10). Both observations are consistent with a scenario in which trigger factor, by hydrophobic interaction, binds to nascent polypeptide chains to assist their folding. Here, we tested whether TF (1-144) and TF (1-247) are capable of forming salt-resistant complexes with translating ribosomes. Trigger factor fragments were added at a final concentration of 1 μ M to *lacZ* *in vitro* transcription/translation reactions. The endogenous full length trigger factor of the cell-free system was calculated to be present at a concentration of 0.2–0.4 μ M during transcription/translation (5) and served as a positive binding control. Fig. 6 shows the presence of trigger factor and its fragments in the respective ribosomal fractions. Accordingly, TF (1-144) and TF (1-247) differ from full-length trigger factor in that their binding to ribosomes at low salt concentration is insensitive to puromycin treatment. In addition, these fragments cannot form salt-resistant complexes with translating ribosomes as observed for the full-length protein. We conclude that the amino-terminal fragment with or without the adjacent FKBP domain is insufficient to allow salt-resistant and puromycin-sensitive binding to ribosomes in the transcription/translation assay. Neither of the fragments TF (145–432), TF (145–247), and TF (248–432), even at concentrations higher than 1 μ M, showed significant cofractionation with ribosomes in the transcription/translation assay (data not shown).

The Amino-terminal 118 Amino Acids of Trigger Factor Form a Compactly Folded Domain—We previously used limited proteolysis by proteinase K and endoproteinase Glu-C (V8) to dissect structural domains of trigger factor (12). Using the full-length protein, we failed to populate stable fragments except for the central FKBP-type PPIase domain. In the course of limited proteolysis of recombinant trigger factor fragments by proteinase K, however, we noticed the appearance of distinct products for TF (1-247) and TF (1-144) (Fig. 7). Within the first 2 min of digestion, TF (1-247) was degraded into two prominent proteolytic fragments (PK-1 and PK-3). By amino-terminal sequencing, PK-1 was identified as an extended PPI-

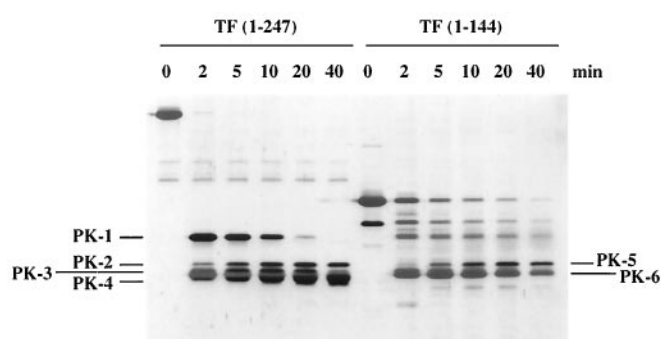


FIG. 7. Time course of limited proteolysis of TF (1-247) and TF (1-144) by proteinase K. Proteolytic fragments were separated by SDS-PAGE and silver stained. PK-1 to PK-6 denote proteolytic fragments that were identified by amino-terminal sequencing and, in part, mass spectroscopy as follows. PK-1, G₁₁₉LEAI; PK-2, V₃SVET, 12,807 Da; PK-3, M₄RGSM, 13,498 Da; PK-4, R₁₄₅KQQA; PK-5, V₃SVET, 12,803 Da; PK-6, M₄RGSM, 13,497 Da. Negative sequence positions refer to the four vector-encoded amino acids MRGS preceding the amino-terminal methionine of trigger factor. Note that PK-2 and PK-5 migrate slower in SDS-PAGE than products PK-3 and PK-6 although being amino-terminally shortened by six additional amino acids.

ase domain starting with glycine 119. We had noticed this cleavage site before when sequencing a transient proteinase K fragment of native, full-length trigger factor (12). With time, this species was further trimmed amino-terminally to give the ~12-kDa FKBP fragment (PK-4) starting at arginine 145. PK-3 started with the amino acids MRGSMQVSV and, therefore, represents the amino terminus of TF (1-247). A comigrating species (PK-6), starting with the same amino-terminal amino acids, was generated by proteolysis of TF (1-144). Thus, amino acids 1–144 of trigger factor contain a compactly folded domain. To determine the carboxyl terminus of this domain, mass spectroscopy was performed with TF (1-247) and TF (1-144) samples that had been digested with proteinase K and passed over a reversed-phase HPLC column. The predominant proteolytic fragment common to TF (1-144) and TF (1-247) had an experimentally determined molecular mass of 13,497–13,498 Da. Taking into account that the vector encoded four additional amino acids at the amino terminus, this mass perfectly fits to a fragment of trigger factor ending with glutamine 118 and having a theoretical mass of 13,498.6. This is in agreement with the sequencing result for the larger proteolytic fragment of TF (1-247), PK-1, starting with glycine 119. A second proteolytic fragment (PK-2 and PK-5) was common to TF (1-247) and TF (1-144). Surprisingly, these polypeptides, although migrating slower in SDS-PAGE than PK-3 and PK-6, arose from further proteolysis of PK-3 and PK-6 by six amino-terminal amino acids as revealed by amino-terminal sequencing and mass spectroscopy. Together, amino acids 1–118 (or 3–118) comprise a compactly folded domain of *E. coli* trigger factor.

TF (1-118) Is Capable of Binding to Ribosomes—To test whether the above described structural element of trigger factor, encompassing the amino-terminal 118 amino acids, also represents a functional entity, two experimental approaches were taken: (a) TF (1-118) was constructed genetically and purified as a recombinant fragment by Ni²⁺-NTA affinity chromatography; and (b) a preparative digest of TF (1-247) was carried out to produce the proteinase K fragments PK-1 (starting with glycine 119) and PK-3 (ending with glutamine 118). TF (1-118) and the proteolytic fragments of TF (1-247) were incubated with equimolar amounts of purified ribosomes followed by sucrose cushion centrifugation to re-isolate the ribosomal particles (Fig. 8a). SDS-PAGE analysis of the ribosomal fractions by staining with Coomassie Brilliant Blue was insuf-

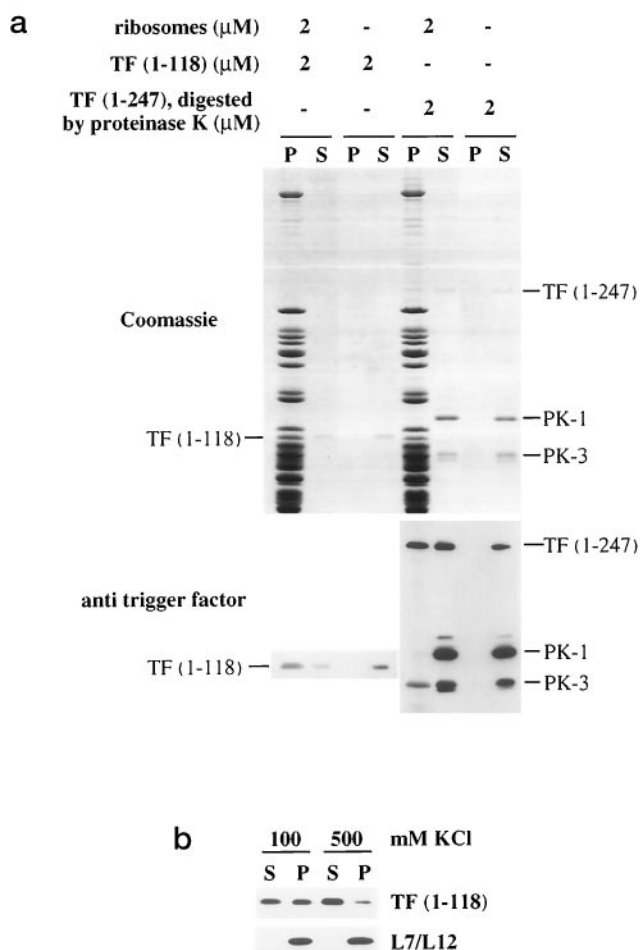


FIG. 8. TF (1-118) is the ribosome-binding domain of trigger factor. *a*, TF (1-118) was prepared both as a recombinant His-tagged fragment as well as the proteolytic fragment PK-3 of TF (1-247), followed by incubation with equimolar amounts of purified ribosomes. Upon sucrose cushion centrifugation, pellet (*P*) and supernatant (*S*) fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining or immunoblotting with a trigger factor-specific antiserum. *b*, DH5 α cells overproducing recombinant TF (1-118) were harvested, lysed, and applied to sucrose cushion centrifugation in presence of 100 or 500 mM KCl, respectively. Ribosomal pellets (*P*) and postribosomal supernatants (*S*) were analyzed by immunoblotting with a trigger factor-specific antiserum and, as a control, antiserum specific for ribosomal L7/L12. Note that only 50% of the supernatant fractions were loaded.

sufficient to identify the trigger factor fragments, because they comigrated with ribosomal proteins. Therefore, these fractions were additionally subjected to immunoblotting using a trigger factor-specific antiserum (Fig. 8*a*). Positive ribosome-binding signals were obtained for recombinant TF (1-118), residual TF (1-247), and the PK-3 fragment, representing the amino-terminal 118 amino acids of TF (1-247). In control incubations lacking ribosomes, all fragments were recovered from the supernatant fractions. Furthermore, PK-1 exclusively remained in the supernatant fractions, even in presence of ribosomes. Thus, the presence of TF (1-118) and PK-3 in pellet fractions demonstrates ribosome binding by these fragments *in vitro*. Recombinant TF (1-118), in addition, was capable of binding to ribosomes *in vivo*, as demonstrated by its cosedimentation with ribosomal particles prepared from cell extracts of TF (1-118) overproducing cells (Fig. 8*b*). We conclude that TF (1-118) not only structurally but also functionally fulfills the criteria of a novel trigger factor domain that mediates binding to ribosomes *in vivo* and *in vitro*.

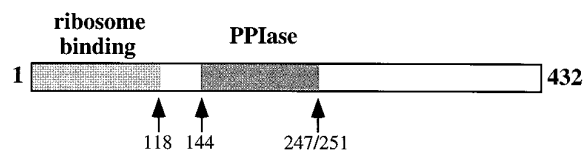


FIG. 9. Schematic representation of the domain structure of trigger factor. Identified domains are drawn to scale.

DISCUSSION

The present study led to the identification of the ribosome binding site of trigger factor. We found that the amino-terminal trigger factor fragment TF (1-144): (i) is necessary and sufficient for ribosome binding *in vivo* and *in vitro*; (ii) shares the same binding site with full-length trigger factor on the large ribosomal subunit; and (iii) contains a compactly folded, protease-protected domain comprising the amino-terminal 118 amino acids of trigger factor. This proteolytic fragment was capable of binding to ribosomes *in vivo* and *in vitro* and, therefore, represents the second structural and functional element of trigger factor described so far (Fig. 9). Unlike the central PPIase domain, which belongs to the FKBP family of PPIases (10, 12, 14), homology searches against updated data bases did not reveal any protein with similarity to TF (1-118). These findings do not formally exclude the existence of additional sites within trigger factor that contribute to ribosome binding but by themselves are insufficient for binding to ribosomes. Such sites would be difficult to detect experimentally.

Based on limited proteolysis, the amino-terminal ribosome-binding domain and the central FKBP domain are separated by an exposed 26-amino acid peptide linker that is accessible to proteinase K and endoproteinase Glu-C (V8) (this study and Ref. 12). The PhD program for prediction of secondary structure and surface accessibility in proteins (17) predicted a surface-exposed loop conformation for amino acids 118-136 of *E. coli* and *Haemophilus influenzae* trigger factor. With proteinase K, the initial proteolytic cleavage within trigger factor occurs between glutamine 118 and glycine 119, followed by rather slow cleavage between leucine 144 and arginine 145. We, therefore, envision this 26-amino acid linker to be part of an extended PPIase domain rather than being part of the ribosome binding domain. An extended PPIase fragment, starting with glycine 119, was observed before upon digestion of the full-length trigger factor (12). The functional significance of this extension is unclear. In our previous study, TF (1-118) was not observed as a stable proteinase K fragment for the following reason. TF (1-118) essentially comigrates in SDS-PAGE with the PPIase domain fragment TF (145-247). The overall resistance to proteolysis of TF (1-118) is lower compared with that of the FKBP domain, explaining why TF (1-118) escaped the detection in the former study, in particular because longer digestion times were used. Stoller *et al.* (13) noticed an amino-terminal fragment of ~14 kDa upon digestion of trigger factor by subtilisin. Interestingly, in their study, ribosome-bound trigger factor was completely resistant to subtilisin treatment, indicating the occurrence of substantial structural rearrangements upon ribosome binding or a protection from proteolytic attack by the ribosomal particle. In agreement with our work, the amino-terminal portion of trigger factor was also very sensitive to digestion by subtilisin (13).

Concerning the function of trigger factor at the ribosome, a crucial finding of this study is that a recombinant fragment comprising both the ribosome binding domain and the PPIase domain, TF (1-247), does not form puromycin-sensitive and salt-resistant complexes with translating ribosomes. For this function, apparently, the entire trigger factor molecule is required. Given that the salt-resistant complex of full-length

trigger factor with translating ribosomes is disrupted by puromycin treatment, it is tempting to assume that a trigger factor-nascent chain interaction is the basis of the tight ribosomal association. Accordingly, salt-resistant binding of trigger factor to ribosomes would reflect a nonionic interaction with nascent polypeptide chains. Folding assistance could be achieved by a cooperative process involving the catalytic power of the PPIase domain and an additional high affinity substrate binding reminiscent of molecular chaperone action (8). The isolated PPIase domain is deficient in high affinity binding of protein substrates (8), which may account for the failure of TF (1–247) to interact with translating ribosomes in a salt-resistant and puromycin-sensitive fashion. High affinity substrate binding by trigger factor could rely on a functional interaction of the FKBP domain with the carboxyl-terminal portion of its polypeptide chain and, possibly, additional contributions by the amino-terminal domain. It is evident, however, that the amino-terminal trigger factor domain fulfills the important targeting function to a site on the large ribosomal particle that is in proximity to the emerging nascent chain. This targeting function is likely to be a prerequisite for the efficient association of trigger factor with nascent chains.

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