Identification of the prolyl isomerase domain of *Escherichia coli* trigger factor

Thomas Hesterkamp, Bernd Bukau*

Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

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Abstract ***E. coli*** **trigger factor is a protein of 48 kDa which was recently identified as a ribosome-bound peptidyl-prolyl-*cis/trans*-isomerase (PPIase) capable of catalysing protein folding in vitro. We found trigger factor in association with nascent polypeptide chains, suggesting a function in the co-translational folding of proteins. Sequence comparisons revealed a homology of a segment of trigger factor with PPIases of the FK506 binding protein (FKBP) family. Here, we report on the purification of trigger factor and a domain assignment of its polypeptide chain by microsequencing and mass spectroscopy of proteolytic fragments. Two proteases generated fragments of 12-13 kDa molecular weight that encompass the predicted FKBP domain and possess PPIase activity in vitro. Sequence alignment of the known trigger factor proteins demonstrates a high degree of conservation within this central functional domain of the protein.**

Key words: Chaperone; Protein folding; Ribosome; Proteolysis; Nascent polypeptide chain

1. Introduction

Over the last decade compelling evidence has accumulated that protein folding in the cell is assisted by molecular chaperones and folding enzymes. Folding enzymes catalyse disulfide bond formation and isomerisation of Xaa-prolyl peptide bonds and thereby accelerate rate-limiting folding steps [1,2].

E. coli trigger factor was originally identified by its activity to stimulate membrane translocation of the precursor of the outer membrane protein A (proOmpA) in vitro. Based on its stoichiometric binding to this substrate it was proposed to act as a secretion specific chaperone [3,4]. However, subsequent investigations showed that cells depleted of trigger factor had no secretion defect for proOmpA [5]. New interest in trigger factor resulted from recent work of several laboratories. Rahfeld et al. isolated a ribosome-bound PPIase and identified it as trigger factor. Importantly, they showed that trigger factor can accelerate a rate-limiting, prolyl-isomerisation dependent step in refolding of a denatured RNase T1 mutant protein in vitro [6]. The PPIase activity of trigger factor has been confirmed by an independent study [7]. Using a coupled transcription/translation system synthesizing β-galactosidase, Hesterkamp et al. identified trigger factor as a nascent polypeptide chain associated protein of *E. coli* [7]. Furthermore, analysing the interactions of *E. coli* proteins with secretory and non-secretory nascent polypeptide chains by photocrosslinking, Valent et al. [8] and Hesterkamp et al. [7] identified a major crosslinking component as trigger factor. Goldberg and coworkers found trigger factor in complex with the GroEL chaperone and demonstrated a role of trigger factor in GroEL-dependent degradation of an unfolded protein [9]. Together, these studies implicate trigger factor in the folding of nascent polypeptide chains, possibly by virtue of its PPIase activity, and raise the possibility that it provides a link to the protein folding machinery composed of molecular chaperones.

Performing a database analysis for trigger factor homologs, we noticed a restricted homology of a central part of its polypeptide chain with PPIases of the FKBP family [7]. Residues conserved between trigger factor and the eukaryotic FKBP12 include the aliphatic and aromatic residues which form the hydrophobic core of the substrate binding pocket of FKBP12 [10-12]. Independently, Callebaut and Mornon reported on the same homology [13].

We approached the domain organization of trigger factor biochemically by limited proteolysis of the native protein. Two proteases generated fragments encompassing the predicted FKBP domain. These fragments are shown to possess the same specific activity as full length trigger factor, thus identifying the central domain of trigger factor from V_{132} to E_{247} as the PPIase.

2. Materials and methods

2.1. Materials

Arabinose was from Sigma; proteinase K, endoproteinase Glu-C (V8), Pefabloc SC, and TLCK were from Boehringer Mannheim; DEAE-Sepharose Fast Flow and Superdex 200 were from Pharmacia; Bio-Gel HT hydroxyapatite was from Biorad; Protein Pak 8 HR was from Waters; Suc-Ala-Phe-Pro-Phe-pNA was from Bachem.

2.2. Purification of trigger factor

A 6 l culture of the trigger factor overproducing strain DH5α pTIG2 [5] was grown in LB medium supplemented with ampicillin (50 μg/ml) at 37°C to an OD_{600} of 0.3. To induce expression of the *tig* gene, arabinose was added to 0.2% (w/v) final concentration and growth was continued until an OD_{600} of 1.8 was reached. The culture was cooled in an ice-water bath and all subsequent steps were performed at 4°C. Cells were harvested by centrifugation and washed with buffer A (20 mM Tris-Cl, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.5 at 4°C), 50 mM NaCl. Cells were lysed by a single passage through a French press at 8000 lb/inch^2 followed by 30 min centrifugation at 30000×*g*. The cleared supernatant was then subjected to a second centrifugation at 250000×*g* for 2 h. This supernatant was applied onto a DEAE-Sepharose FF column (250 ml) equilibrated in buffer A, 50 mM NaCl and the bound proteins were eluted by a linear gradient of 50 to 500 mM NaCl in buffer A. Trigger factor containing fractions were pooled, diluted 1:4 with buffer A, applied onto a Protein Pak 8 HR column equilibrated in buffer A, 50 mM NaCl and eluted as before. Trigger factor peak fractions were extensively dialysed against 50 mM Na-phosphate buffer, pH 7.0 and loaded onto a Bio-Gel HT hydroxyapatite column (50 ml). Trigger factor eluted in a linear phosphate gradient at around 280 mM Na-phosphate. Trigger factor containing fractions were collected, concentrated 15-fold, loaded onto a Superdex 200 (26/60) column equilibrated in buffer

*Corresponding author. Fax: (49) (6221) 545892.
E-mail: bukau@sun0.urz.uni-heidelberg.de

Abbreviations: PPIase, peptidyl-prolyl-*cis/trans*-isomerase; FKBP, FK506 binding protein; proOmpA, precursor of the outer membrane protein A.

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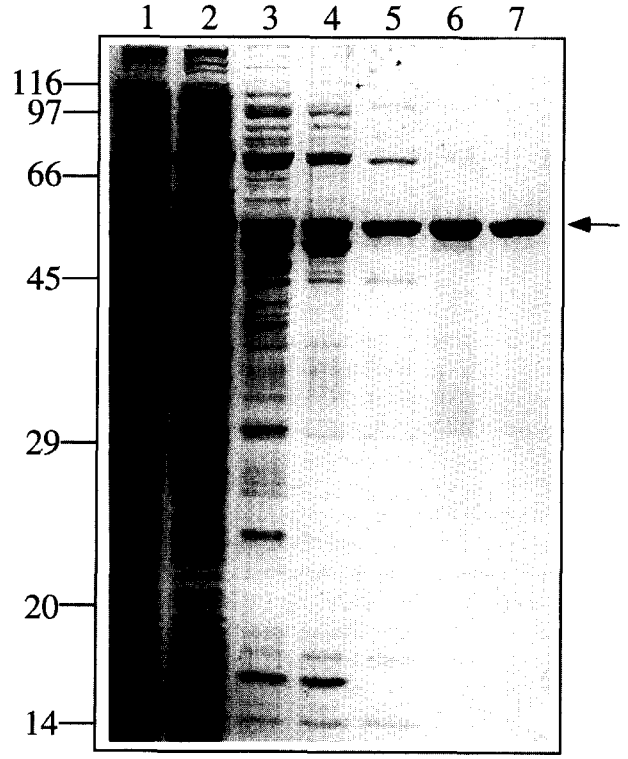

Fig. 1. Coomassie-stained denaturing polyacrylamide gel showing trigger factor purification steps; lanes: 1, cells prior to *tig* induction; 2, cells 2 h after *tig* induction; 3, proteins after DEAE-Sepharose Fast Flow; 4, proteins after first Protein Pak 8 HR run; 5, proteins after Bio-Gel HT hydroxyapatite; 6, after Superdex 200; 7, purified trigger factor after second Protein Pak 8 HR run. The arrow indicates the migration position of trigger factor.

A, 50 mM NaCl and eluted at 1 ml/min. Peak fractions were collected and reconcentrated on the Protein Pak 8 HR column as outlined before. The final preparation was dialysed against buffer A, 100 mM NaCl, 5% (v/v) glycerol, frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined by the Bradford assay using lysozyme as standard.

2.3. Limited proteolysis and preparation of trigger factor fragments

Trigger factor was diluted to 0.8 mg/ml with 20 mM Tris-Cl, 2 mM $CaCl_2$, pH 8.0 and incubated at 30°C with proteinase K at a mass ratio of 600:1. Aliquots taken at the indicated time points were mixed with Pefabloc SC and prepared for SDS-PAGE analysis (13.5% acrylamide). Endoproteinase Glu-C (V8) digests were performed as above except that the substrate:protease ratio was 80:1. Proteolysis was stopped by addition of TLCK to 0.5 mM final concentration and analysed as above. Proteins were detected by silver staining. A preparative endoproteinase Glu-C (V8) digest of 5 mg of trigger factor was carried out at 37°C for 10 min at a substrate:protease ratio of 60:1. TLCK was added to 1 mM followed by 10-fold dilution of the proteins with ice-cold buffer A. The protein was applied onto the Protein Pak 8 HR column and eluted by a linear gradient of 0–500 mM NaCl. Fractions were analysed by SDS-PAGE and Coomassie staining. Relevant fractions were frozen in small aliquots in liquid nitrogen and stored at −80°C. Further incubation of individual column fractions for 30 min at 30°C did not change the protein pattern, indicating the absence of active protease. N-terminal sequences were determined on a Applied Biosystems 473A Sequencer, mass spectroscopy was performed with a Kratos Compact MALDI 3 V4.0.

2.4. PPIase assay

Prolyl isomerase activities were measured as triplicates essentially as described in [6] on a Varian DMS 200 spectrophotometer at 10°C with the oligopeptide Suc-Ala-Phe-Pro-Phe-pNA as substrate. First order rate constants were calculated using the ENZFIT software (Elsevier Biosoft). The apparent specificity constants $(k_{cat}/K_m)_{app}$ were determined as outlined in [6].

2.5. Sequence alignment

Sequences for members of the trigger factor family were taken from databases (*E. coli* and *H. influenzae* trigger factor Swiss-Prot accession numbers P22257 and P44837, respectively, *C. jejuni* trigger factor genebank identifier cjdnatig, and *M. genitalium* GSDB accession number L43967) and aligned within the MegAlign software package using the Clustal algorithm and default settings.

3. Results

3.1. Purification of trigger factor

In order to study the structure-function relationship of *E. coli* trigger factor we established a new protocol for the preparative-scale purification of the native protein. For this purpose, strain DH5α bearing a multi-copy plasmid with the

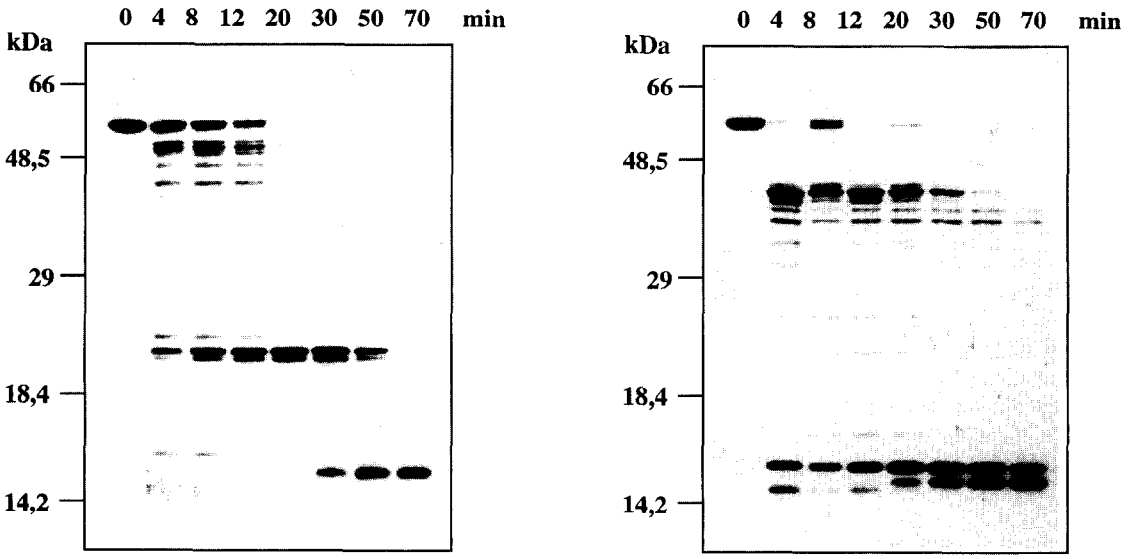

Fig. 2. Silver-stained denaturing polyacrylamide gels showing the time courses of limited proteolysis of native trigger factor with proteinase K (a) and endoproteinase Glu-C (V8) (b); variations in the amount of full length protein within the time course (b) are due to incomplete inactivation of the protease in the respective samples.

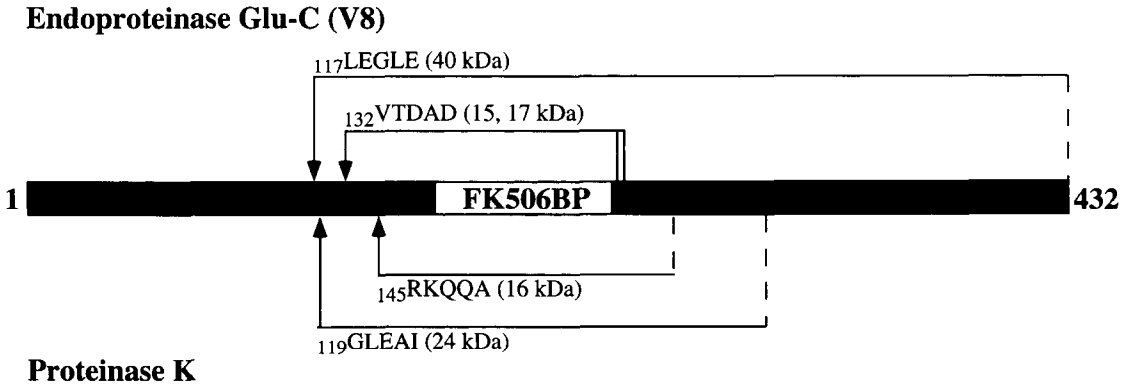

Fig. 3. Schematic representation of the trigger factor polypeptide chain; proteolytic fragments are drawn to scale, dashed lines indicate assumed C-terminal boundaries of fragments according to their apparent molecular weights, and the central stretch with predicted FKBP homology is unshaded.

entire *tig* gene under control of the *araB* promoter (pTIG2) [5] was used. We purified the protein by five column chromatography steps as outlined in section 2 and shown in Fig. 1. The final purification step gave a protein that was essentially free of contaminating proteins as judged by Coomassie staining of the denaturing gel. The yield was 35 mg trigger factor from a 6 l culture. The apparent specificity constant $(k_{cat}/K_m)_{app}$ for the PPIase substrate Suc-Ala-Phe-Pro-Phe-pNA was 0.88 ± 0.1 μM^{-1} s^{-1} and thus is in good agreement with the specific activities reported before [6,8].

3.2. Limited proteolysis of trigger factor

To investigate the domain organization of trigger factor's polypeptide chain, limited proteolysis using papain, proteinase K, trypsin, and endoproteinase Glu-C (V8) was carried out. While papain and trypsin failed to generate stable trigger factor fragments at different substrate to protease ratios (not shown), proteinase K and endoproteinase Glu-C (V8) digests resulted in distinct proteolytic fragments (Fig. 2a,b). Proteinase K degraded trigger factor to fragments of apparent molecular weights of 24 and 16 kDa. Endoproteinase Glu-C (V8) generated fragments of apparent molecular weights of 40, 17, and 15 kDa. Prolonged incubation with this protease led to a complete conversion of the 17 kDa species into the 15 kDa species (not shown). All major fragments were N-terminally sequenced. In Fig. 3 the sequences obtained are depicted on a linear map of the polypeptide chain. Evidently, both low molecular weight fragments of the endoproteinase Glu-C (V8) digest start with V_{132} and thus must be trimmed differently at the C-terminus. The N-termini of all sequenced fragments cluster in a stretch of around 30 amino acids (positions 117–145) preceding the part of the polypeptide chain that has homology to FKBPs.

3.3. Preparation of endoproteinase Glu-C (V8) fragments

Proteinase K and endoproteinase Glu-C (V8) generated small fragments that were likely to encompass the predicted FKBP-homology domain. To directly test our prediction we performed a preparative endoproteinase Glu-C (V8) digest followed by fractionation of the fragments by ion exchange chromatography. A separation of the 15 and 17 kDa species from the 40 kDa fragment was achieved (Fig. 4, upper panel). Furthermore, all fractions were devoid of full length trigger factor. The lower panel of Fig. 4 shows the corresponding PPIase activities of identical aliquots of the column fractions. Clearly, all fractions containing either of the low molecular weight fragments display PPIase activity and the activities correlate with the relative amounts of fragments as judged by their staining intensities. Furthermore, the fractions containing the 40 kDa fragment display PPIase activity. The comparatively lower activity of these fractions is explained by the lower molar amounts of the 40 kDa species compared to the 15 and 17 kDa species. We determined the exact size of the

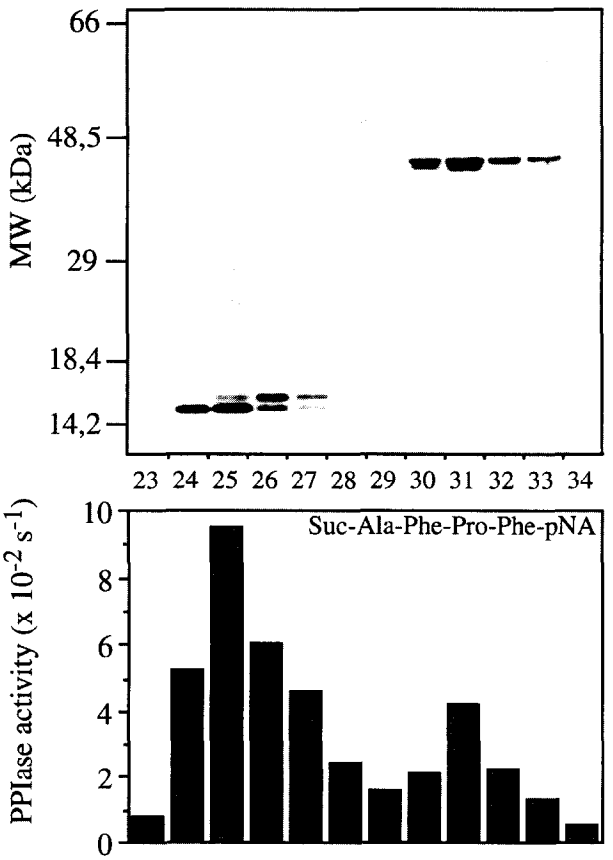

Fig. 4. Separation of endoproteinase Glu-C (V8) derived trigger factor fragments by ion exchange chromatography; Coomassie-stained denaturing polyacrylamide gel (upper panel) and corresponding PPIase activity measurements (lower panel).

Table 1. Size of trigger factor fragments and specific PPIase activities

Apparent MW (Da), SDS-PAGE	Exp. determined MW (Da), mass spectroscopy	Deduced fragment boundaries	Predicted MW (Da)	Specific PPIase activity, $(k_{cat}/K_m)_{app}$ (μM^{-1} s^{-1})
15000	12787.6	V_{132}–E_{247}	12779.6	0.75 ± 0.06[a]
17000	13217.1	V_{132}–E_{251}	13194.1	n.d.
40000	n.d.	L_{117}–A_{432}[b]	35217.8[b]	0.67 ± 0.1[c]
58000[d]	n.d.	M_1–A_{432}	48026.5	0.88 ± 0.1

[a]Fraction 24, Fig. 4.
[b]The trigger factor fragment of 40000 Da apparent molecular weight was assumed to encompass the entire C-terminus of the protein.
[c]Fraction 31, Fig. 4.
[d]This species corresponds to full length trigger factor.

fragments by mass spectroscopy. Table 1 lists the experimentally determined molecular masses of the fragments, the deduced C-terminal boundaries, the predicted molecular masses for the deduced fragments, and specific PPIase activities. Accordingly, the smallest proteolytic fragment with a molecular mass of 12779.6 Da has the same specific activity as full length trigger factor.

3.4. Sequence alignment of trigger factor sequences from different species

To identify conserved sequence elements within the trigger factor family the sequences of all four published homologs were compared (Fig. 5). The overall amino acid identities with the *E. coli* protein are 64.4% within 432 amino acid overlap for the *H. influenzae* protein, 25.3% within 423 amino acid overlap for *M. genitalium*, and 31.6% within 410 amino acid overlap for the larger form of *C. jejuni* trigger factor. The highest identity between the four sequences is found within the PPIase domain. According to our alignment, amino acid identities with the V_{132}–E_{247} fragment of *E. coli* are 77.6% for *H. influenzae*, 29.3% for *M. genitalium*, and 44.8% for *C. jejuni*. A second, highly conserved stretch of approx. 20 amino acids (A_{36}–V_{54} for *E. coli* trigger factor) can be identified in the N-terminus of the proteins. The functional importance of this region is unknown. The C-terminal third of the proteins is the least conserved part. A set of hydrophobic and aromatic amino acids aligned by the Clustal algorithm, namely A_{341}, V_{345}, I_{371}, A_{375}, Y_{378}, and L_{394} for the *E. coli* protein, may fulfil structural roles in this part of the polypeptide chain.

4. Discussion

The identification of trigger factor in association with nascent polypeptide chains [7,8], together with the demonstration that trigger factor is as a PPIase [6,7] which can accelerate protein folding more efficiently than the other PPIases tested so far [6], sheds new light on the mechanism of protein folding in the cytosol. Prolyl isomerisation might be catalysed directly at the place of protein synthesis and thus be an early event in the folding of proteins.

To address the domain organisation of trigger factor biochemically, we subjected it to limited proteolysis. Two proteases generated highly populated fragments encompassing a central segment which was identified by sequence comparisons to have restricted homology with PPIases of the FKBP family [7,13]. For endoproteinase Glu-C (V8) digested trigger factor a 12.8 kDa fragment (V_{132}–E_{247}), strikingly similar in size to the archetype FKBP12, is shown to display a PPIase activity that is as high as the activity of full length trigger factor. These findings experimentally confirm our homology-derived domain assignment. In addition, the sequence identity of the four known trigger factor proteins is highest within the sub-

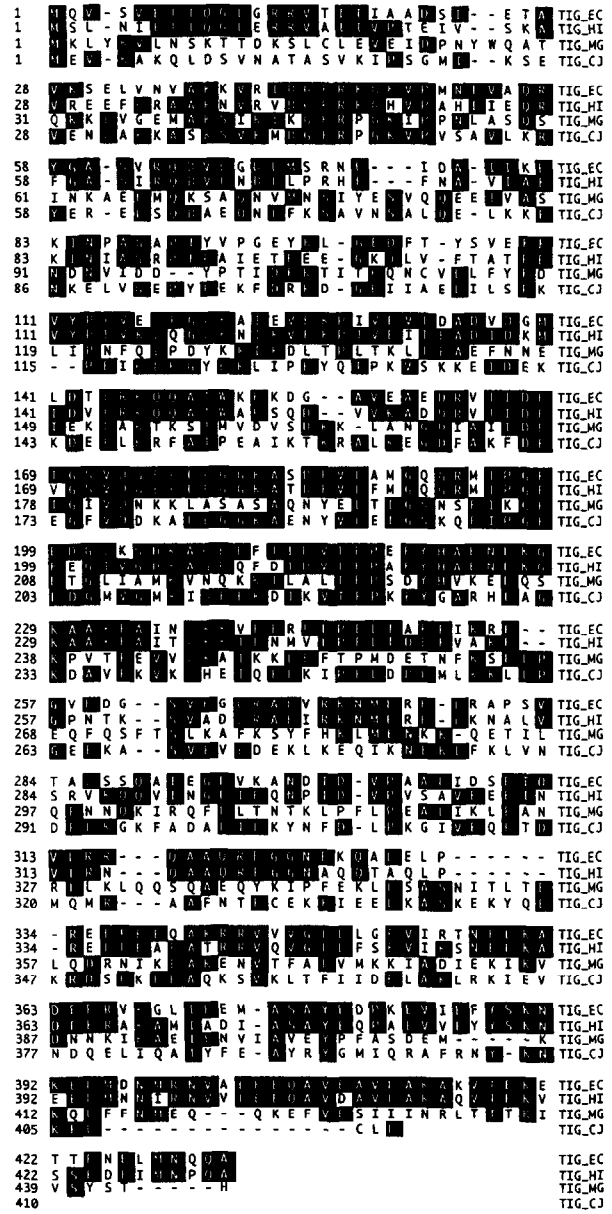

Fig. 5. Sequence alignment of the four known members of the trigger factor family; Sequences are: EC, *Escherichia coli*; HI, *Haemophilus influenzae*; MG, *Mycoplasma genitalium*; CJ, *Campylobacter jejuni*, large form. Sequence identity in at least two sequences is indicated by black boxes.

strate binding pocket of the FKBP domain. It thus appears that the PPIase activity is a central feature of the trigger factor family.

The failure to inhibit trigger factor's PPIase with FK506 [6] may be due to a predicted structural divergence from the FKBP12 archetype in two loop regions [13], or to single amino acid exchanges within the substrate binding pocket. Of the five FKBP12 residues known to be involved in hydrogen bond formation with the inhibitor [12], three are identical or conservatively exchanged in trigger factor (FKBP12 I_{56}-trigger factor I_{195}, Y_{82}–Y_{221}, D_{37}–E_{178}) and two are non-conservative exchanges (Q_{53}–G_{192}, E_{54}–R_{193}). Thus, an altered hydrogen bonding capacity within trigger factor's substrate binding pocket may account for its insensitivity towards FK506.

Two further activities of trigger factor, namely stable stoichiometric substrate binding [3,4], which is not assumed to be a function of the PPIase, and ribosome binding [14] await a structure-function analysis. It will be interesting to see whether the domains located N- and C-terminal to the PPIase domain fulfil these functions.

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