The EMBO Journal vol.14 no.23 pp.6021–6027, 1995

Trigger factor is involved in GroEL-dependent protein degradation in *Escherichia coli* and promotes binding of GroEL to unfolded proteins

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In *Escherichia coli*, the molecular chaperones of hsp60/hsp10 (GroEL/GroES) families are required not only for protein folding but also for the rapid degradation of certain abnormal proteins. The rate-limiting step in the degradation of the fusion protein CRAG by protease ClpP appears to be the formation of a complex with GroEL. We have isolated these complexes and found that each GroEL 14mer contained a short-lived fragment of CRAG plus a 50 kDa polypeptide, which we identified by sequencing and immunological methods as Trigger Factor (TF). Upon ATP addition, GroEL and TF dissociated together from CRAG but remained tightly associated with each other even upon gel filtration. TF was originally proposed to function in protein translocation across membranes but altering cellular content of TF did not affect this process *in vivo*. By contrast, low levels of TF expression markedly reduced CRAG degradation, while an overproduction of TF greatly stimulated this process. Furthermore, in extracts of cells expressing high levels of TF, the capacity of GroEL to bind to CRAG is greatly increased. Overproduction of TF also stimulated GroEL's ability to bind to other unfolded proteins (fetuin and histone). Thus, TF is a rate-limiting factor for CRAG degradation; it appears to regulate GroEL function and to promote the formation of TF–GroEL–CRAG complexes which are critical for proteolysis.

Keywords: chaperone/*Escherichia coli*/GroEL/proteolysis

Introduction

In prokaryotic and eukaryotic cells, proteins with highly abnormal conformations are degraded rapidly by an ATP-requiring process (Goldberg, 1992). In *Escherichia coli*, the degradation of certain abnormal proteins has been shown to require both an ATP-dependent protease, Lon (La) or Clp (Ti) and also the molecular chaperones of the hsp70 and hsp60 families (Keller and Simon, 1988; Straus *et al.*, 1988; Tilly *et al.*, 1989; Sherman and Goldberg, 1992; Kandror *et al.*, 1994). Molecular chaperones play essential roles in protein folding, assembly of multimeric complexes and translocation across membranes (Pelham, 1986; Hartl, 1991; Welsh, 1991; Gething and Sambrook, 1992). However, if the chaperones fail to promote the proper folding or assembly of a protein, as may occur with a mutant or damaged polypeptide, they apparently can promote the rapid degradation of the non-functional polypeptides. For example, DnaK (the Hsp70 homolog in bacteria) and its cofactors, DnaJ and GrpE, form complexes with the short-lived mutant protein, PhoA61, which appear to be essential for the rapid hydrolysis of PhoA61 by proteases La (Sherman and Goldberg, 1992a) or Clp (unpublished observation). By contrast, the short-lived fusion protein CRAG (Hellebust *et al.*, 1990; Sherman and Goldberg, 1991) requires GroEL (the Hsp60 homolog) and GroES for its rapid degradation (Kandror *et al.*, 1994).

This unstable protein contains a short unfolded domain of the cro-repressor and of β-galactosidase flanking at either end a domain of protein A. The presence of the protein A domain allows rapid isolation of CRAG from cell extracts using an IgG column. When expressed in cells, CRAG forms complexes *in vivo* with GroEL and DnaK. Although this association with DnaK was found to be unimportant for degradation, the formation of the complex with GroEL correlated closely with the rate of CRAG hydrolysis. Futhermore, overexpression of GroEL with or without its cofactor, GroES, stimulated CRAG breakdown, while ts mutations in the *groES* gene blocked CRAG degradation at the non-permissive temperature (Kandror *et al.*, 1994).

Despite extensive efforts, we have failed thus far to reconstitute CRAG degradation in cell extracts or with the addition of ATP, purified GroEL, GroES and protease ClpP, the latter being responsible for most CRAG breakdown *in vivo* (Kandror *et al.*, 1994). This failure to demonstrate degradation *in vitro* under any of the conditions tested, raised the possibility that other proteins may also be important for CRAG degradation. Therefore, we searched for additional polypeptides that might be associated with the complex of CRAG and GroEL and might also function in the degradative process.

In this paper, we report the unexpected discovery of an additional component of these complexes, which we identified as Trigger Factor (TF). This polypeptide was originally isolated by Wickner and co-workers (Crooke and Wickner, 1987; Lecker *et al.*, 1989) because of its ability (shared by the chaperones GroEL and SecB) to bind to certain unfolded polypeptides and to promote their translocation into membrane vesicles. It was therefore proposed to function as a molecular chaperone in protein translocation. Genetic studies, however, indicated that TF was not necessary for this translocation process *in vivo* (Guthrie and Wickner, 1990) and thus, its physiological role is uncertain. Evidence is presented here that TF is associated with GroEL *in vivo*, that it is an important cofactor in GroEL-dependent CRAG degradation and that it can regulate the capacity of GroEL to associate with unfolded proteins.

Results

CRAG–GroEL complexes

To identify new components of the CRAG–GroEL complexes, we prepared cell extracts and isolated CRAG,

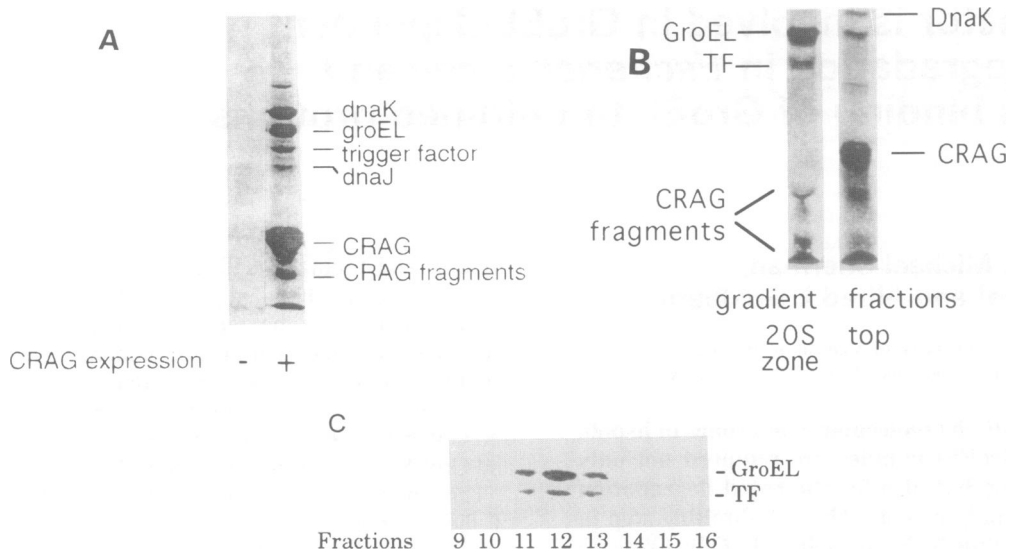

Fig. 1. Purification of complexes containing GroEL, TF and CRAG fragments. (**A**) Isolation of CRAG-associated proteins on an IgG column. *Escherichia coli* strain C600 transformed with pRIT2 plasmid, carrying the CRAG gene and the control C600 cells were grown and labeled as described in Materials and methods. Cell extracts were loaded onto the IgG–Sepharose column. CRAG and the associated proteins, which bound to the IgG column, were eluted with acetic acid and analyzed by SDS–PAGE and autoradiography. (**B**) Separation of GroEL-containing complexes of CRAG by sucrose gradient centrifugation. 100 μl of ^{35}S-labeled C600 extract, containing 60 μg of protein ($1–2\times10^6$ c.p.m./μg) were loaded onto a 5 ml linear sucrose gradient (15–30%) and centrifuged at 200 000 *g* for 2 h at 4°C. Fractions from the 20S zone, containing GroEL, were collected, combined and loaded onto a 2 ml IgG–Sepharose column. CRAG and the associated proteins were eluted with acetic acid and analyzed by SDS–PAGE and autoradiography. The fractions from the top of the gradient were also loaded on the IgG column, eluted with acid and analyzed by similar procedures. (**C**) ATP causes the release of a GroEL–TF complex from CRAG. The GroEL–TF–CRAG complex was isolated by sucrose gradient centrifugation and affinity chromatography on the IgG–Sepharose column. Then the chaperones associated with CRAG was eluted with ATP. The proteins from the ATP eluate were subjected to gel filtration on a Superose 6 column, in presence of ATP and sequential fractions from this column were analyzed by SDS–PAGE. GroEL and TF in the fractions were detected by Western blot with anti-GroEL and anti-TF antibodies.

together with the associated polypeptides, by affinity chromatography. Since the CRAG molecule contains a protein A domain (Hellebust *et al.*, 1990), it can be isolated with an IgG–Sepharose column, along with the associated chaperones (Sherman and Goldberg, 1991, 1992a,b, 1993; Kandror *et al.*, 1994). We then eluted the bound proteins with acid and analyzed them by SDS–PAGE (Kandror *et al.*, 1994). Several polypeptides were eluted with CRAG (Figure 1A), including GroEL, DnaK and DnaJ, plus several minor unidentified proteins (Sherman and Goldberg, 1991).

Since GroEL has a native M_r of 700 kDa, CRAG bound to GroEL could be easily separated from free CRAG and low molecular weight complexes that contained DnaK, by sucrose gradient centrifugation (Figure 1B) or gel filtration (data not shown). The fractions containing GroEL were then pooled and loaded onto an IgG–Sepharose column to isolate the CRAG–GroEL complexes. When this bound material was eluted and analyzed by SDS–PAGE, the GroEL–CRAG complexes, to our surprise, did not contain full-length CRAG but only smaller CRAG fragments (Figure 1B). We identified these bands as CRAG fragments, since they reacted on Western blot with anti-β-galactosidase antibody and also with non-immune serum and thus contained segments of protein A (not shown). Moreover, N-terminal microsequencing of these fragments showed that both of them contained the same N-terminus as the full-length CRAG molecule. Therefore, they seem to have been formed by proteolytic cleavage of the C-terminal region. In unpublished studies (manuscript in preparation), we have found that these GroEL-bound fragments are short-lived intermediates in CRAG degradation. Thus, these complexes are intimately involved in the proteolytic pathway.

A similar approach using IgG–Sepharose was used to isolate free CRAG and DnaK-bound CRAG from the low molecular weight fractions of the sucrose gradient (Figure 1B). Previous studies have shown that *dnaK* deletion does not reduce the rate of CRAG degradation (Kandror *et al.*, 1994) and thus, this association of some CRAG with DnaK is not related to degradation. In contrast to the polypeptides associated with GroEL, the IgG-binding material in the lower molecular weight fractions from the sucrose gradient was primarily full length CRAG (Figure 1B).

TF is in complexes with GroEL and CRAG fragments

After purification by affinity chromatography, the GroEL complexes were found to contain, in addition to CRAG fragments, a distinct polypeptide band of 50 kDa (Figure 1B). When this polypeptide was micro-sequenced, its first 10 N-terminal residues (MQVSVETTQG) perfectly matched the N-terminus of TF. Furthermore, this band reacted specifically on a Western blot with an anti-TF antibody kindly provided by Dr W. Wickner (not shown). TF was originally isolated by Wickner and co-workers because of its ability to promote translocation of certain polypeptides into membrane vesicles (Crooke and Wickner, 1987; Lecker *et al.*, 1989). However, because strains with low levels of TF showed no defects in protein translocation (Guthrie and Wickner, 1990), its function *in vivo* has remained unclear.

In extracts of *E.coli* not expressing CRAG, neither

GroEL nor TF bound to the IgG column (Figure 1A). Therefore, these polypeptides must bind only through their association with the CRAG fragments. Either there exists a ternary GroEL–TF–CRAG complex or there are separate binary GroEL–CRAG and TF–CRAG complexes. Several observations indicate the existence of the ternary complexes. When the GroEL fraction from the sucrose gradient was applied onto the IgG column, GroEL and TF both bound and when ATP was added to elute the bound GroEL, both proteins were eluted from CRAG. Furthermore, after elution with ATP, GroEL and TF remained tightly associated with each other, even through gel filtration (Figure 1C). Since the cells were labeled uniformly with ^{35}S and since the number of methionine residues in these proteins is known, we calculated the ratio of the components in these complexes to be ~1 GroEL 14mer:1 CRAG:1 TF monomer.

These findings raised the possibility that TF and GroEL bind together to CRAG (or other proteins) and are then eluted as a complex upon ATP addition. To determine whether TF is associated in the cell only with GroEL molecules that are bound to CRAG, we tested in extracts of cells that do not express CRAG, whether TF could be co-precipitated with an anti-GroEL antibody and whether GroEL could be co-immunoprecipitated with an anti-TF antibody. These experiments indicated that a part of the total GroEL population in wild-type cells is associated with TF. Moreover, as shown in Figure 2A and B, the presence of CRAG in the extracts did not influence the amount of TF co-immunoprecipitated with GroEL. It seems likely, therefore, that TF first forms a complex with GroEL, which then associates with CRAG or with other unfolded proteins (see below).

Influence of TF content on CRAG degradation

Since TF is found in these 'degradative complexes' containing GroEL and CRAG fragments, we tested whether TF plays a role in the GroEL-dependent degradation of CRAG. Since TF is essential for viability, we studied a strain kindly provided by Dr Wickner that expresses TF at either normal or very low levels depending on the growth conditions (Guthrie and Wickner, 1990). This strain had the *tig* gene integrated into the chromosome, under the control of the *ara* promotor. Therefore, when grown in medium containing arabinose, this strain expressed TF at high levels. However, when grown in medium containing glucose instead of arabinose, TF synthesis was repressed and after 6 h of growth on this medium, the level of TF fell by more than 90% (Guthrie and Wickner, 1990). Interestingly, the strain with the low level of TF expression was not viable upon expression of CRAG and we were unable to transform it with the usual CRAG plasmid. To allow transformation with the CRAG-expressing plasmid, we first transformed the TF-underexpressor strain with the plasmid containing a repressor that prevented CRAG expression at normal temperatures (c1857 repressor). We then transformed this strain with the pMS421 plasmid containing CRAG under the control of the Pr promotor. To induce CRAG, the cells were shifted for 5 min to 42°C and degradation was then measured at different temperatures. In the TF-deficient cells, the degradation rate was 2-fold lower than in the wild-type at 42°C (Figure 3A and D) or at 37°C (not

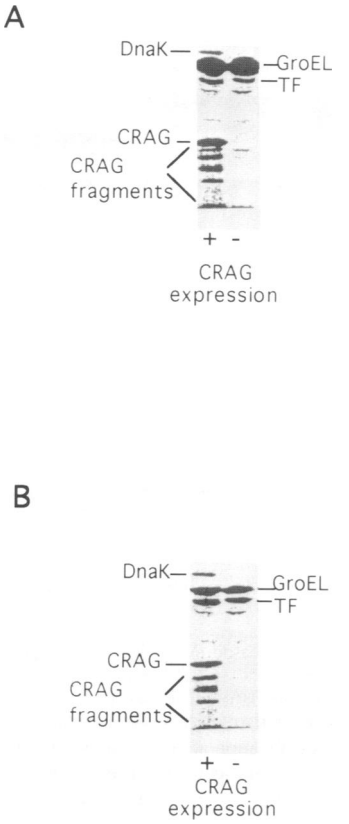

Fig. 2. A fraction of GroEL in the wild-type cells exists in complex with TF. C600 cells were labeled uniformly and the cell extracts were prepared as described in Materials and methods. 50 µl of ^{35}S-labeled extract, containing 30 µg of protein ($1–2\times10^6$ c.p.m./µg) was diluted to 1 ml with the immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.5). Then 2 µl of either anti-GroEL **(A)** or anti-TF **(B)** antibodies and 20 µl of protein A immobilized on Trisacryl (Pierce) were added. The mixture was incubated for 2 h at 4°C with rotation and then centrifuged, after which the pellets were washed three times with 1 ml of immunoprecipitation buffer containing 0.1% SDS and subjected to SDS–PAGE. Gels were dried and the amount of radioactive proteins was determined using a PhosphorImager.

shown). However, if cells were incubated at 20°C, the rate of CRAG degradation in TF-deficient cells was 5- to 6-fold lower (Figure 3B–D). Under these conditions, TF was present at ~5–7% of the level in wild-type cells, as measured by Western blot with an anti-TF antibody (data not shown).

These findings suggested that TF may be a rate-limiting factor in the breakdown of CRAG. To test this hypothesis, we used a TF-overexpressing strain, which had the *tig* gene on a multicopy plasmid (pTIG2) under the regulation of the *ara* promotor (Guthrie and Wickner, 1990). When TF was overexpressed, very little full-length CRAG was found in the cell; instead, the predominant species of CRAG was a relatively stable 25 kDa fragment (Figure 3A and C). The small amount of the full-length CRAG and the appearance of the 25 kDa fragment could reflect the extremely rapid proteolytic conversion of the translated protein into this smaller polypeptide. Alternatively, this polypeptide may have been generated by the premature termination of CRAG transcription or translation upon overproduction of TF [such a function of TF in translation

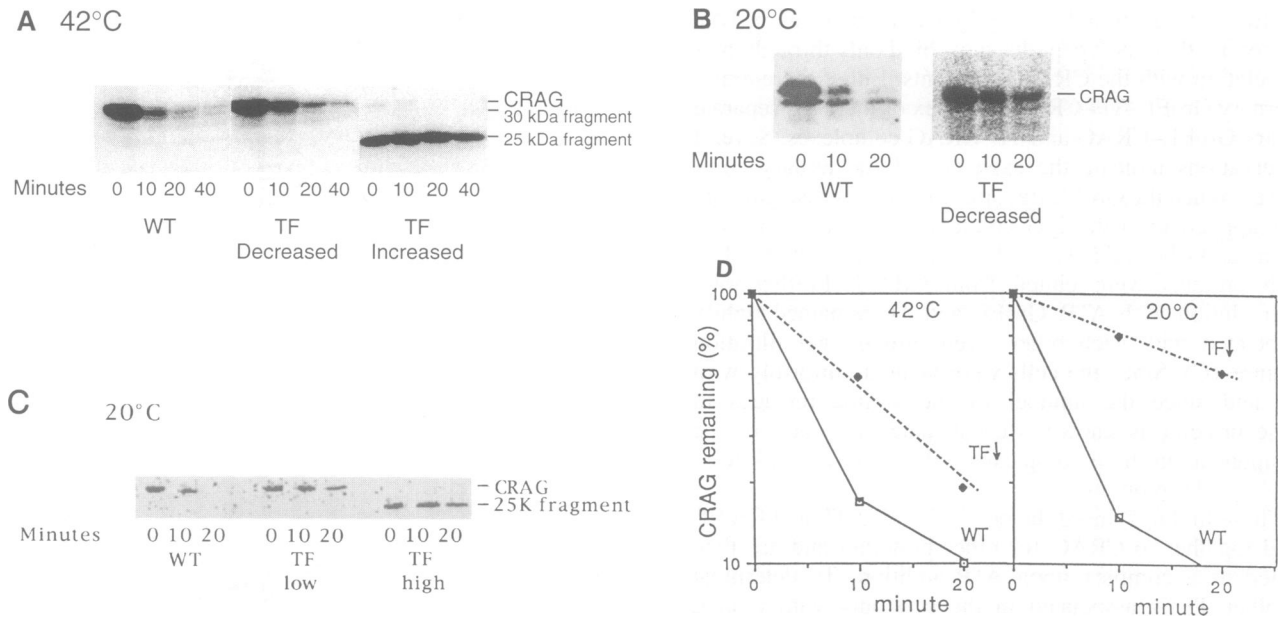

Fig. 3. The effect of increased and reduced TF expression on CRAG degradation. TF-overproducing, TF-underproducing and C600 wild-type strains were grown for 6 h at 35°C in LB, which was supplemented with glucose to inhibit *tig* expression or with arabinose to induce maximal *tig* expression. CRAG degradation was then measured at different temperatures. (**A**) CRAG degradation at 42°C. A portion of each culture was shifted for 5 min to 42°C for CRAG expression and the rate of CRAG degradation was then measured at 42°C by Western blot with ^{125}I-labelled protein A. (**B** and **C**) CRAG degradation at 20°C. To measure the rate of CRAG degradation at 20°C, the portion of exponentially growing cell cultures were shifted to 20°C for 2 h. Subsequently, after 5 min incubation at 42°C for CRAG induction, they were immediately cooled to 20°C and CRAG degradation was measured at this temperature by Western blot (B) or by a pulse–chase experiment followed by immunoprecipitation (C) (see Materials and methods). (**D**) Quantitation of degradation rates at 42°C and 20°C. In the wild-type, the rates of CRAG degradation were similar in either the glucose- or the arabinose-containing medium.

seemed possible in light of TF's reported association with ribosomes (Lill *et al.*, 1988)].

To decide between these possibilities, we introduced into the TF-overexpressor strain a *lon-clpP* double mutation, which completely blocks degradation of CRAG in normal cells (Kandror *et al.*, 1994). In this strain, full-length CRAG accumulated and very little of the 25 kDa fragment was found (Figure 4). Thus, the high level of TF accelerated the proteolytic breakdown of newly synthesized CRAG to the 25 kDa fragment and this accelerated proteolysis requires the same proteases as those that function in wild-type cells in CRAG breakdown. This 25 kDa fragment was probably more stable under these conditions because some components other than TF had become rate-limiting for its further degradation. In any case, since increased levels of TF accelerated CRAG breakdown, while decreased levels slowed degradation, TF is clearly a rate-limiting component in the degradation of this (and presumably also of other) abnormal proteins.

TF enhances GroEL's ability to bind to CRAG and other unfolded proteins

Theoretically, TF overexpression may promote CRAG degradation by stimulating the association of GroEL with this abnormal protein or by somehow altering the nature of the chaperone–CRAG complex so as to favor the degradative process. To decide between these possible roles of TF in CRAG degradation, we have tested whether the ability of GroEL to bind to CRAG varies under conditions of differential TF expression.

For this experiment, we prepared radiolabeled extracts from wild-type cells and from cells producing TF in very high or very low levels. The extracts were then loaded onto identical affinity columns containing immobilized CRAG as the ligand and the GroEL that bound was then eluted with 1 mM ATP. A similar approach has been used previously in our laboratory to study the regulation of GroEL's binding capacity (Sherman and Goldberg, 1991, 1992a,b, 1993). Although the level of TF expression did not alter the total GroEL content in the cells (data not shown), it dramatically affected the amount of GroEL that became associated with the CRAG column (Figure 5A). A significant amount of GroEL in the extracts from the TF-overexpressor strain bound to the column, while very little GroEL bound from the wild-type extracts and no binding was seen when the TF-underexpressing strain was used. Thus, increasing the level of TF expression in the cell enhanced GroEL's ability to bind to CRAG (Figure 5A) and greatly stimulated the rate of CRAG degradation (Figure 2).

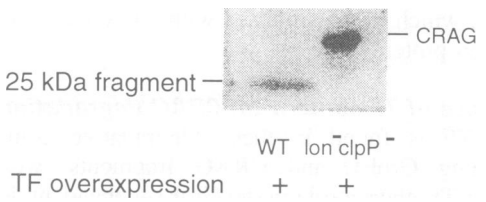

Fig. 4. The *lon-clpP* double mutation prevented CRAG degradation despite high levels of TF. Wild-type C600 and *lon-clpP* mutant cells, both carrying the TF-overexpressing plasmid and the CRAG plasmid, were grown in LB with arabinose for 4 h at 35°C. Then, an aliquot from each strain was precipitated with 10% TCA and analyzed by Western blot with an anti-β-galactosidase antibody and ^{125}I-labelled protein A.

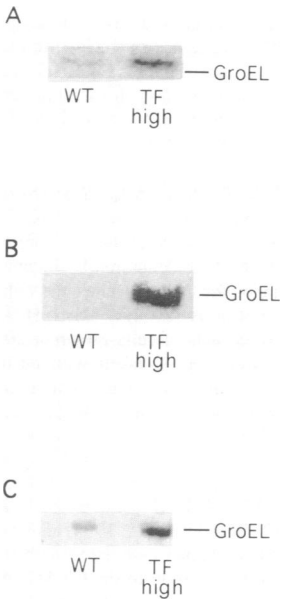

Fig. 5. Increased cellular TF content stimulates binding of GroEL to immobilized CRAG (**A**), fetuin (**B**) and histone (**C**). Same amounts of ^{35}S-labeled (A) or non-radioactive (B and C) extracts from the wild-type or TF-overexpressing cells were applied onto affinity columns containing CRAG (A), fetuin (B) or histone (C) as the ligands (see Materials and methods). The amount of the bound GroEL was determined after ATP-elution by SDS–PAGE followed by Western blot (B and C) or autoradiography (A).

To test whether TF expression could also promote GroEL's capacity to bind to other unfolded polypeptides, we passed the extracts of control and TF-overexpressor cells over the columns containing urea-denatured histone or fetuin as a ligand (Sherman and Goldberg, 1994). Overproduction of TF greatly enhanced GroEL's ability to bind to these denatured polypeptides (Figure 5B and C), as was observed with CRAG. Thus, TF appears to regulate GroEL's affinity for different unfolded proteins and, therefore, may function as a co-chaperone with GroEL not only in protein degradation but also in other processes (e.g. protein folding).

Discussion

Although TF is a major protein in *E.coli*, its function *in vivo* has long been unclear. Based on its ability to promote translocation of proOmpA into membrane vesicles, Wickner and co-workers originally proposed that it functions as a molecular chaperone in protein secretion (Crooke and Wickner, 1987; Lecker *et al.*, 1989). The finding of TF together with GroEL and CRAG fragments in the 'degradative complexes' was totally unexpected and led to the demonstration that TF plays a critical role in CRAG breakdown. This involvement of TF in the degradative process may also account in part for our previous failure to demonstrate GroEL-dependent proteolysis in cell-free extracts. Studies are in progress to determine whether this process involves additional components and whether TF plays a role in degradation of other cell proteins.

Both TF and GroEL appear to be rate-limiting factors in CRAG degradation. Decreased expression of TF in growing cells reduced CRAG degradation 2- to 6-fold depending on the growth temperature. This marked inhibition was incomplete probably because TF was still present at least at 5–7% of the wild-type level in the TF-deficient strain, as measured by the Western blot with anti-TF antibody. It is also noteworthy that transformation of TF-deficient cells with the plasmid that generated high levels of CRAG expression was lethal. In other words, CRAG could only be tolerated in cells producing normal or increased levels of TF. A similar toxicity upon CRAG expression was seen with a *groEL-clpP* double mutant, in which CRAG degradation was blocked. Thus, GroEL, GroES, TF and the protease, all appear important for cell viability in the presense of large amounts of CRAG, presumably because they function together in the rapid degradation of this polypeptide and possibly, other abnormal proteins (Straus *et al.*, 1988).

A dramatic result was seen upon overexpression of TF which accelerated CRAG breakdown to such an extent that the intact CRAG molecule was present only in trace amounts. Instead, a new more stable proteolytic fragment was formed in this strain. The reasons for its generation in large amounts and its relative stability are uncertain but it appears likely that TF, if overexpressed, binds to CRAG and influences the accessability of some parts of the substrate to proteases La or Clp, while blocking others. In any case, it is noteworthy that CRAG degradation can be accelerated by overproduction of TF or GroEL (Kandror *et al.*, 1994), both of which enhance its breakdown by the same proteases as those that function in wild-type cells.

Several genes involved in protein degradation including *clpP*, *clpX* and *lon* are adjacent to each other on the *E.coli* chromosome (*E.coli* linkage map). Interestingly, the *tig* gene is localized in the exact same region next to *clpP*. Although the *tig* gene has its own promotor, its localization close to genes coding for proteases may be important for their coordinate regulation, for example, during the cell cycle, when at certain stages, gene copy number may be doubled.

A primary role of GroEL is to catalyze the proper assembly of proteins. However, with many abnormal polypeptides (e.g. CRAG), successful folding may be impossible and GroEL together with TF may therefore promote their rapid breakdown. These studies demonstrate that the formation of a complex between GroEL–TF and CRAG seems to be an essential step for its breakdown. It is noteworthy that a fraction of GroEL in wild-type cells exists in complexes with TF. Thus, this association does not require the presence of CRAG. Moreover, these two proteins dissociated together from CRAG upon addition of ATP and remained tightly associated with each other. It appears very likely that TF expression stimulates CRAG degradation, at least in part, by promoting the association of GroEL with this polypeptide. Increasing or decreasing the expression of TF in the cell altered GroEL's capacity to bind to CRAG and the rate of CRAG degradation in similar ways. Moreover, TF overexpression dramatically enhanced GroEL's binding to other denatured polypeptides. Thus, TF appears to function as a co-chaperone together with GroEL. There is no reason to assume that this new function of TF as a regulator of GroEL's binding capacity is restricted to proteolysis. By stimulating GroEL's association with unfolded proteins, TF can prob-

ably also promote the folding and translocation of certain polypeptides.

It is possible that TF not only promotes substrate binding by GroEL but also favors proteolytic digestion of CRAG by some additional mechanism. Recent studies from G.Fisher and co-workers indicated that TF also possesses a peptidyl–prolyl isomerase activity (PPI) (G.Fisher, personal communication). This finding may suggest a possible mechanism for TF's stimulation of GroEL's binding capacity: TF associated with GroEL may alter substrate conformation by isomerizing a critical proline residue and thus, may facilitate its entering into GroEL's central cavity. This process may be equally important for both protein folding and protein degradation. It is presently unclear what determines the further fate of the TF–GroEL–bound protein and whether the chaperone(s) will promote the proper folding or the degradation of the bound polypeptide.

Materials and methods

Cell growth and labelling of cell proteins

To express CRAG, C600 *E.coli* strain was transformed with pRIT2 plasmid, carrying CRAG gene (Hellebust *et al.*, 1990). To increase the expression of TF, *E.coli* C600 strain was transformed with pTIG2 plasmid (kindly provided by Dr W.Wickner), which carries the *tig* gene under the regulation of an arabinose (ara) promotor. To fully induce the *tig* gene, cells were grown on LB supplemented with 0.2% arabinose for 6 h at 35°C. These growth conditions led to more than a 10-fold increase in the production of TF over that seen without arabinose. To decrease the level of TF in the cell, the *tig* gene under the regulation of the ara promotor was inserted into the chromosome by transduction into C600. These cells were grown for 6 h at 35°C in LB supplemented with glucose to inhibit *tig* expression. After 6 h, the level of TF fell by >90% below the levels in LB alone.

To label cell proteins uniformly (Figure 1B), the wild-type cells were grown at 35°C in Minimal Salt Davis (Difco) supplemented with thiamine, 0.5% glucose and all amino acids except methionine, valine and serine. At early log-phase 0.5 mCi of trans-S-label, a mixture of [^{35}S]cysteine and [^{35}S]methionine, (ICN) (>1000Ci/mmol) and 3×10^{-5} M of non-radioactive methionine were added to 100 ml of the culture for 30 min to label cell proteins. Cells were harvested by centrifugation and resuspended in 50 mM Tris, 5 mM EDTA, 2 mg/ml lysozyme, pH 8.0. This suspension was frozen, thawed and then subjected to brief sonication. After centrifugation at 14 000 *g* for 10 min to remove cell debris, the soluble cell extract was used in further experiments.

For *in vitro* studies of GroEL binding to CRAG columns the TF-overexpressing, TF-underexpressing and wild-type cells were grown in LB at 35°C until mid-log phase and then shifted to 20°C for 2 h. [^{35}S]methionine (ICN, 100 μCi/ml) was added and after 30 min of labeling, the cell extracts were prepared as described above.

Determination of the rate of CRAG degradation

The rate of CRAG degradation in the cells was measured by Western blot or by the pulse–chase protocol and subsequent immunoprecipitation of CRAG (Kandror *et al.*, 1994). Both approaches gave similar results (see Figure 2). The pulse–chase experiments, like all other studies, were done in LB. The cells were grown at 20°C, [^{35}S]methionine (ICN, 100 μCi/ml) was added immediately before the cells were shifted to 42°C to induce CRAG expression. After a 5 min pulse, non-radioactive methionine (final concentration 0.3 mM) and chloramphenicol (0.1 mg/ml) were added to prevent re-incorporation of ^{35}S into proteins. Aliquots of 1 ml were taken at different time points, the cells were pelleted, boiled in 0.3% SDS and subjected to immunoprecipitation with anti-β-galactosidase antibody as described previously (Kandror *et al.*, 1994).

IgG–Sepharose column

IgG–Sepharose column (Sigma) was equilibrated with buffer A (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT). Crude extract of ^{35}S-labeled cell (50 mg at $1–2\times10^6$ c.p.m./mg) or combined fractions from the sucrose gradient were applied to 2 ml columns, which were then washed with buffer A until the radioactivity in the eluted material had fallen to $5–8\times10^3$c.p.m./ml (~20 column volumes). The chaperones were eluted from CRAG with 1 mM ATP and 2 mM MgCl in buffer A. To elute all the proteins from the column (including CRAG) 100 mM acetic acid (pH 2.5) was used. The proteins in each fraction were precipitated with 10% TCA, washed with acetone and analyzed by SDS–PAGE followed by autoradiograhpy or by Western blot.

In vitro binding of GroEL to unfolded proteins

Fetuin–agarose and histone–agarose were purchased from Sigma Chemical Co. Two ml protein–agarose columns were prepared and the bound proteins (fetuin or histone) were denatured by incubation with 6 M urea and 1 M mercaptoethanol for 20 min. Then the columns were washed and equilibrated with buffer A (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT). 50 mg of extracts of cells grown at 20°C were applied to 2 ml columns, which were then washed with buffer A (~20 column volumes). The bound proteins were eluted with 1 mM ATP and 2 mM MgCl in buffer A. The proteins in each fraction were precipitated with 10% TCA, washed with acetone and analyzed either by SDS–PAGE and Western blot with anti-GroEL antibody and [^{125}I]protein A.

The CRAG column was prepared as described previously (Sherman and Goldberg, 1991). When CRAG is expressed in *E.coli*, a fraction of it exists bound to GroEL (Kandror *et al.*, 1994) and even after purification of CRAG, some GroEL remained associated with it. Therefore, to test the effects of TF on GroEL binding to the CRAG column, radiolabeled cell extracts were used. 20 ml of the wild-type, TF-underexpressing and TF-overexpressing cells were labeled with [^{35}S] methionine in LB and the extracts were prepared as described above. The extracts (50 mg at $1–2\times10^5$ c.p.m./mg) were applied onto identical 2 ml columns. The columns were washed and the bound proteins were eluted from the columns as described above. The presence of GroEL in the eluate was determined by autoradiography.

Sucrose gradient centrifugation

After removal of the cell debris by centrifugation at 14 000 *g* for 10 min, 60 μg (by protein) of a soluble cell extract ($1–2\times10^6$ c.p.m./mg) was loaded onto a 5 ml linear sucrose gradient (15–30% sucrose in TE buffer) and centrifuged at 200 000 *g* for 4 h at 4°C. Fractions from the 20S zone were collected, combined and immediately loaded onto IgG column (2 ml volume).

Acknowledgements

We are very grateful to Dr W.Wickner for valuable reagents and to Ms Aurora Scott for assistance in the preparation of this manuscript. These studies were made possible by grants from AMGEN and the National Institutes of Health to A.L.G. and a Postdoctoral Fellowship to M.S. from The Medical Foundation.

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Received on July 17, 1995; revised on September 15, 1995