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Co-translational Binding of GroEL to Nascent Polypeptides Is Followed by Post-translational Encapsulation by GroES to Mediate Protein Folding*

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The eubacterial chaperonins GroEL and GroES are essential chaperones and primarily assist protein folding in the cell. Although the molecular mechanism of the GroEL system has been examined previously, the mechanism by which GroEL and GroES assist folding of nascent polypeptides during translation is still poorly understood. We previously demonstrated a co-translational involvement of the *Escherichia coli* GroEL in folding of newly synthesized polypeptides using a reconstituted cell-free translation system (Ying, B. W., Taguchi, H., Kondo, M., and Ueda, T. (2005) *J. Biol. Chem.* 280, 12035–12040). Employing the same system here, we further characterized the mechanism by which GroEL assists folding of translated proteins via encapsulation into the GroEL-GroES cavity. The stable co-translational association between GroEL and the newly synthesized polypeptide is dependent on the length of the nascent chain. Furthermore, GroES is capable of interacting with the GroEL-nascent peptide-ribosome complex, and experiments using a single-ring variant of GroEL clearly indicate that GroES association occurs only at the *trans*-ring, not the *cis*-ring, of GroEL. GroEL holds the nascent chain on the ribosome in a polypeptide length-dependent manner and post-translationally encapsulates the polypeptide using the GroES cap to accomplish the chaperonin-mediated folding process.

The *Escherichia coli* chaperonin GroEL is an essential heat shock protein, reaching ~1% of total cytoplasmic proteins (1–5). GroEL supports the folding of a considerable number of proteins with the assistance of the co-chaperonin GroES (6–11). GroEL forms a large ring-shaped structure comprised of two heptameric rings of identical 57-kDa subunits, and these rings are stacked back-to-back (12, 13).

GroEL executes two consecutive processes: binding of substrate proteins to prevent irreversible aggregation (the *holder* function) and release of the arrested protein to complete folding (the *folder* function) (5). The orderly progression of these two reactions is coordinated by ATP and GroES as follows. The substrate polypeptide is bound through multiple apical domains (7) along the inside surface of the GroEL rings (14). ATP-triggered binding of GroES to the substrate-loaded GroEL *cis*-ring leads to a release of the substrate protein. In the most productive pathway, the release of the substrate protein results in its encapsulation in a cavity formed by the GroEL-GroES complex (15–17). Both GroES and the substrate protein contact the *cis*-ring of GroEL but are subsequently detached by the binding of ATP to the *trans*-ring (18).

The mechanistic details of GroEL function in the folding of chemically denatured proteins have been well elucidated (1–5). In *E. coli*, three major chaperone systems, the trigger factor (TF),[3] DnaK, and GroEL systems, are known to be involved in the folding of translated proteins (4, 19). The prevailing view assumes that TF and the DnaK system act as co-translational chaperones to prevent protein aggregation, whereas GroEL acts as a post-translational chaperone to help polypeptides that have been released from the ribosome to reach their native state (4, 19). This model of the roles of the major chaperones is essentially based on the well designed *in vitro* order-of-addition experiments (20), on genetic experiments showing that simultaneous deletion of TF and DnaK is lethal (9, 21), and on the fact that TF is a ribosome-tethered chaperone (22–26). However, the recent finding that overproduction of GroEL and GroES permits the growth of the *E. coli* lacking TF and DnaK (27, 28) has raised questions as to whether the roles of these chaperones are non-overlapping.

We recently reevaluated the role of GroEL in the folding of newly translated polypeptides *in vivo* and *in vitro* and found that GroEL co-translationally associated with nascent polypeptides (29). To investigate the involvement of GroEL during translation, we have used a highly controllable cell-free translation system, called the PURE (protein synthesis using recombinant *e*lements) system (30, 31), that contains only a minimal set of purified *E. coli* factors responsible for protein synthesis. Taking advantage of the PURE system, which lacks all endogenous chaperones (30, 32), we clearly demonstrated that GroEL associated with the translation complex and mediated correct

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[3] The abbreviations used are: TF, trigger factor; PURE, protein synthesis using recombinant elements; DSP, dithiobis(succinimidyl propionate); CCT, chaperonin containing t-complex polypeptide 1; TRiC, TcP-1 ring complex.

folding by encapsulating the newly synthesized polypeptide in the cavity formed by the GroEL-GroES complex (29). How GroEL associates with the translation complex and how GroES participates in the folding process remain to be clarified.

The aim of this study was to elucidate the detailed molecular mechanism of the co-translational involvement of GroEL. The results presented here show that a stable association of a nascent protein with GroEL is dependent on the length of the newly translated polypeptide and that the post-translational capping by GroES leads to encapsulation of the substrate protein into the GroEL-GroES cavity for correct folding.

MATERIALS AND METHODS

Proteins and Reagents—The GroEL variants (GroEL and SR1), GroES, antibodies to each of the proteins, and IgG-horse radish peroxidase conjugates were all obtained from commercial sources or were prepared as described previously (15, 17, 32–34). Dithiobis(succinimidyl propionate) (DSP) was purchased from Pierce.

Construction of Truncated MetK Protein mRNAs—Adenosylmethionine synthetase (MetK) protein mRNAs of various lengths were prepared by PCR amplification using the plasmid pET20b-metK constructed previously (29) as the template. A universal 5′-primer containing a T7 promoter and individual 3′-primers consisting of the corresponding 3′-end MetK sequences and the termination codon sequence TAA were used for PCR amplification to generate the MetK fragments of different lengths.

Cell-free Translation—Transcription translation-coupled cell-free translation using the PURE system was carried out as described (32) with a slight modification of the magnesium concentration (13 mM) in the reaction. When indicated, the final concentrations of the added chaperones were: 0.2 μM GroEL, 14-mer, 0.2 μM GroES, 7-mer, and 0.2 μM SR1, 7-mer.

Autoradiographic Analysis and Enzyme Assays—The solubility of *in vitro* synthesized MetK was evaluated by autoradiographic analysis of the radiolabeled translation products as described previously (32). The solubility of the translated product was calculated as the ratio of the soluble against total amount of ^{35}S-incorporated full-length proteins. MetK activity was assayed as described previously (29). The ratio of the maximal rate of ^{14}C-labeled adenosyl-methionine synthesis to the soluble amount of the translated product was calculated as the relative enzymatic activity of the enzyme.

Western Blot and Proteolysis—Western blotting analyses were performed to detect the presence of GroEL and GroES, as described previously (32). The proteolysis analysis using proteinase K was performed as in the previous report (29).

Preparation of the Translation Complexes—Single-round translation was carried out at 37 °C for ~15 min and quenched by the addition of chilled buffer, as described previously (29). The resultant mixture was immediately separated by centrifugation (35,000 rpm, 5 h) on a 10–50% continuous sucrose gradient and fractionated as described previously (29).

Preparation of the Cross-linked Translation Complexes—A single round of translation was quenched, as described above, if necessary, followed by cross-linking using DSP, according to the manufacturer's protocol. The reaction mixture was then overlaid onto 1.5 ml of buffer G (32) and centrifuged in a TLA100.3 rotor at 50,000 rpm for 4 h. The resulting pellet was dissolved using buffer S (20 mM Hepes-KOH, 10 mM $Mg(OAc)_2$, 30 mM KCl, 7 mM β-mercaptoethanol, pH 7.6).

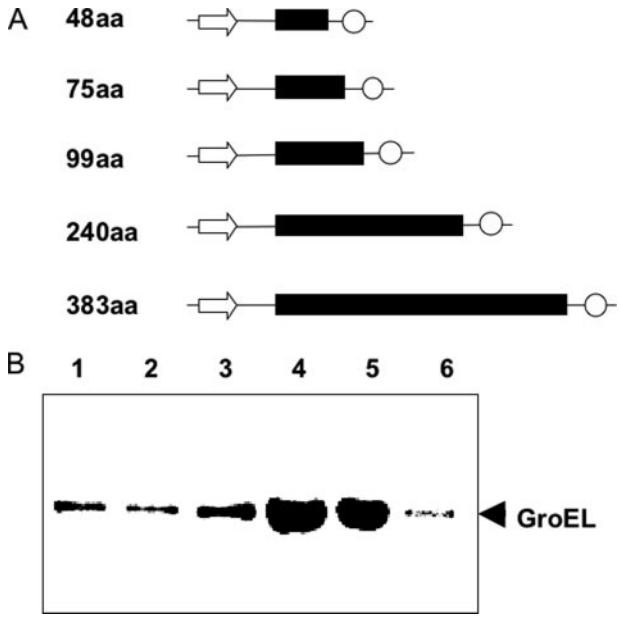

FIGURE 1. **Length-dependence of the co-translational association of GroEL.** *A*, illustration of the mRNAs for MetK variants containing 48, 75, 99, 240, and 383 (full-length) amino acid (*aa*) residues. *Black bars*, MetK fragment; *broad arrows*, T7 promoter; *open circles*, stop codon. *B*, detection of GroEL in the translation complexes. *Lanes 1–5* represent the pellets derived from single-round translation reactions using mRNA for MetK fragments of 48, 75, 99, 240, or 383 amino acids in length. *Lane 6* shows a translation reaction without any mRNA as a negative control. All the translation reactions were carried out in the presence of wild-type GroEL.

RESULTS

Stable Co-translational Association of GroEL with the Nascent MetK Peptide Is Dependent on the Polypeptide Length—We have used the essential *E. coli* protein MetK (35, 36) as a model substrate for further investigation of the translation-coupled folding process since our previous study using the PURE system clearly demonstrated that GroEL was able to associate with the nascent MetK polypeptide on the ribosome (29). In addition, MetK is known to be a GroEL-associated protein, and refolding of denatured MetK is stringently dependent on GroEL, GroES, and ATP (10, 11), indicating that it is an excellent model substrate for the GroEL system.

We first asked whether stable co-translational association of the nascent polypeptide with GroEL required a particular length of polypeptide. Several mRNAs for truncated MetK proteins of various lengths were constructed, as illustrated in Fig. 1*A*. Truncated and full-length (383 amino acids) MetK mRNAs were subsequently subjected to a single round of cell-free translation from which the release factors were omitted. The translation complexes were collected by ultracentrifugation, and the resultant pellets were analyzed by Western blot to detect the presence of GroEL. As shown in Fig. 1*B*, the amount of GroEL in the translation mixture of the 240-amino-acid MetK fragment (*lane 4*) was almost the same as that of the full-length polypeptide (*lane 5*). In contrast, the amount of GroEL in the

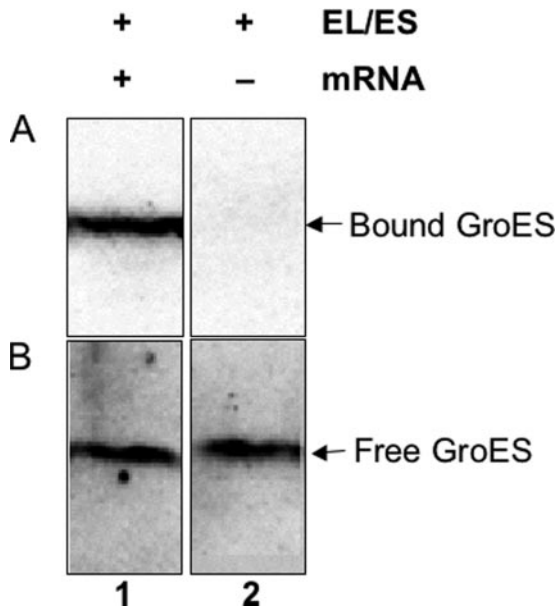

FIGURE 2. **Detection of GroES in the translation complexes.** The pellet (*A*) and supernatant (*B*) following ultracentrifugation (50,000 rpm, 4 h) of the cross-linked translation mixtures were separated by reducing for SDS-PAGE followed by Western blot analysis. Translation was conducted in the presence of GroEL and GroES (+ *EL/ES*) and in the presence (+, *lane 1*) or absence (−, *lane 2*) of the MetK mRNA.

translation complexes markedly decreased in the translation reactions of MetK polypeptides shorter than 99 amino acids (*lanes 1–3*) and was comparable with the background level (*lane 6*). These data suggested that the co-translational association between the nascent polypeptide and GroEL depends on the length of the translated protein.

GroES Associates with the GroEL Translation Complex—We have previously demonstrated that GroES, together with GroEL, encapsulated the MetK polypeptide and was required for its proper folding (29). We next investigated the timing of GroES binding to GroEL during encapsulation of the substrate protein. Considering that the GroEL residues involved in GroES binding mostly overlap with those responsible for substrate protein binding (13, 14, 37, 38), it is unlikely that GroES binds to the *cis*-ring of GroEL, which associates with the nascent peptide on the ribosome. However, recent analyses using single-molecule analysis, as well as GroEL mutants, suggest an intermediate *cis*-ternary complex in which GroES binds to the *cis*-ring of GroEL loaded with the substrate protein during the functional GroEL cycle (34, 39–41). We have previously shown that the translation complex binds GroES *in vivo* (29), although we could not exclude the possibility that GroES co-translationally associated with the translation complex in the cell as the nascent GroES polypeptide emerged from the ribosome. To address this issue, we conducted an *in vitro* translation using the PURE system to examine the co-translational association of GroES.

Single round cell-free translations, with or without the MetK mRNA, were carried out in the presence of both GroEL and GroES followed by ultracentrifugation to isolate the translating ribosome complexes. Both the supernatants (free GroES) and the pellets (GroES bound to the translation complexes) were subjected to Western blot analysis using an antibody against GroES. In the presence of mRNA, GroES was found both in the translating ribosome complex (the pellets) and free in the supernatant (Fig. 2, *lane 1*), whereas GroES was only found as the free form (supernatant) in the absence of mRNA. This result clearly indicates the co-translational involvement of GroES *in vitro* using the PURE system.

Binding Topology of GroES to GroEL Double Rings Using a Single-ring Variant (SR1) of GroEL—The observation that the association of GroES with the translation complex is dependent on the presence of mRNA and the fact that GroES does not directly bind denatured proteins suggest that GroES in the translating ribosome complex may be associated with GroEL bound to the nascent polypeptide. Since GroES can bind either the *cis*-ring or the *trans*-ring of GroEL in the presence of ATP (15), we next investigated the binding topology of the proteins. Since the wild-type GroEL has two identical rings facing back-to-back, it is hard to distinguish which ring contacts GroES. Thus, we utilized a single-ring variant of GroEL, called SR1, to examine GroES binding. SR1 contains four point mutations that abolish the major contacts between the two rings of GroEL and has been shown to bind to, but not readily release, GroES in the presence of ATP (42). If SR1 is able to mediate correct folding of MetK, the presence of GroES in the translation complex would indicate that GroES binds to the *cis*-ring of SR1 because the *trans*-ring is absent in SR1.

To investigate whether SR1 and GroES assist the functional folding of MetK in the PURE system, the solubility and enzymatic activity of MetK synthesized in the presence of SR1 or SR1-GroES were evaluated. The soluble amount of the newly synthesized MetK increased in translation reactions performed with increasing amounts of SR1-GroES or GroEL-GroES (Fig. 3*A*), indicating that SR1-GroES was able to prevent aggregation of the newly translated MetK as efficiently as the GroEL-GroES complex. Subsequently, the enzymatic activity of the newly synthesized MetK was estimated to assess the folding status. The addition of both SR1 and GroES significantly increased the relative biological activity of MetK (Fig. 3*B*). These data indicate that the proper folding of the translated MetK was dependent on SR1-GroES to a similar extent as the wild-type GroEL-GroES.

Subsequently, we ascertained whether SR1 encapsulates MetK into the cavity formed with GroES. Translation products were treated with various concentrations of proteinase K (0, 20, 200 μg/ml) followed by native-PAGE (5–10% gradient gel) to evaluate the resistance of the chaperonin-MetK complex to the protease. As shown in Fig. 3*C*, the products translated in the presence of SR1 and GroES (*lane 1*, *MetK-SR1*) survived proteolysis (*lane 2*, *MetK-SR1*), whereas those translated in the presence of SR1 alone (*lane 4*, *MetK-SR1*) were completely digested by proteinase K, even at the lowest concentration (*lane 5*). In addition, the native and intermediate MetK proteins (Fig. 3*C*, $MetK_I$ and $MetK_N$) were detected as sharp bands, which is consistent with our previous results with wild-type GroEL (29). Thus, SR1 assisted the folding of MetK by forming a *cis*-ternary complex with GroES. Taken together, we conclude that SR1 is able to assist the functional folding of MetK through encapsulation of the

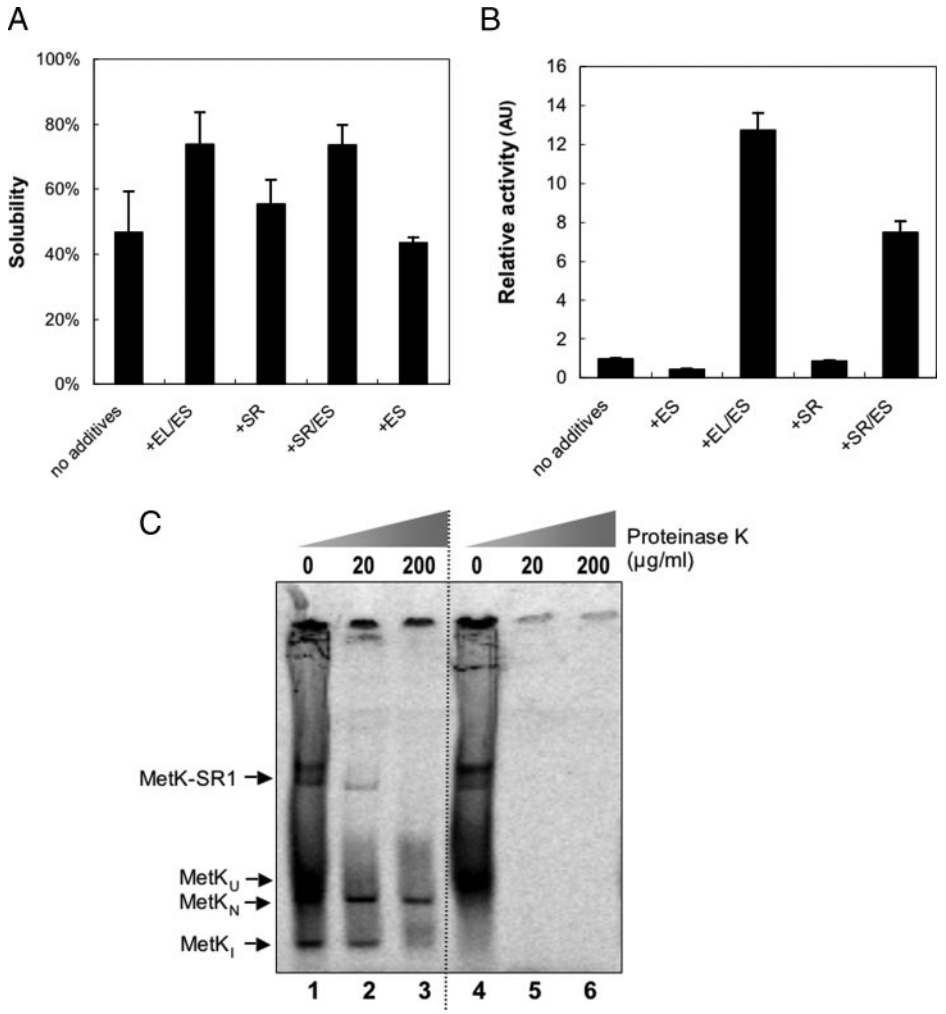

FIGURE 3. **SR1 assists the functional folding of MetK.** *A* and *B*, solubility (*A*) and enzymatic activity (*B*) of MetK translated in the presence (+) or absence (*no additives*) of the indicated chaperonins. +*EL/ES*, GroEL and GroES; +SR, SR1; +SR/ES, SR1 and GroES; AU, arbitrary units. *C*, native-PAGE of the translation products and the proteinase K-digested products. MetK-SR1, *MetK$_U$*, *MetK$_N$*, and *MetK$_I$* indicate SR1 trapped, non-native, native, and intermediate forms of MetK, respectively, according to our previous analysis (29). Cell-free translation of MetK was performed in the presence of SR1 and GroES (*lanes 1–3*) or SR1 alone (*lanes 4–6*). The translation products were digested by 20 μg/ml (*lanes 2* and *4*) or 200 μg/ml (*lanes 3* and *6*) proteinase K. *Lanes 1* and *4* show the translation products without digestion.

newly translated MetK in the cavity formed by the SR1-GroES complex.

GroES Is Absent from the SR1-associated Translation Complex—The interactions among the nascent chain, SR1, and GroES were then investigated. The single-round translation was carried out in the presence of both SR1 and GroES, using the MetK mRNA as the template. The native translation complexes were separated by sucrose density gradient centrifugation, as shown in Fig. 4*A*. The fractions, corresponding to the ribosomal subunits (30S and 50S) and the translating ribosomes (monosomes and polysomes) were analyzed by Western blot. SR1 was evident in the translating ribosomes (*panel b*, monosome and polysome fractions), indicating the co-translational association of SR1 with the nascent MetK. In contrast, the co-chaperonin GroES was hardly detectable in the translation complexes (*panel c*, monosome and polysome fractions) but was found mostly in the upper fractions, probably as a complex with SR1 (*panel c*, fractions of 30S and 50S).

To confirm these results, chemical cross-linking was used to detect possible weak or transient co-translational associations of GroES to SR1 in the translation reactions. Cell-free translation in the presence of SR1 and GroES was performed with or without template mRNA. The translation complexes comprising the ribosome, the nascent peptide, the associated SR1, and GroES, if any, were covalently cross-linked using DSP, a bifunctional cleavable cross-linker targeted for amino groups followed by ultracentrifugation to separate cross-linked translation complexes from the free proteins. Samples of both the initial reaction mixture and the translation complex pellet recovered after ultracentrifugation were separated by gel electrophoresis under reducing conditions to cleave the cross-linked complexes and analyzed by Western blot (Fig. 4*B*). SR1, but not GroES, was detected to a greater extent in the translation complex (*lane 7*), although similar amounts of SR1 and GroES were observed in the reaction mixtures before ultracentrifugation (*lanes 4* and *5*). As controls, SR1 and GroES faintly appeared in the non-translating ribosomes (*lane 8*). Additionally, neither SR1 nor GroES contaminated the PURE system alone (*lanes 3* and *6*). These results clearly demonstrate that GroES is not co-translationally associated with SR1.

DISCUSSION

We previously reported that GroEL co-translationally associated with nascent polypeptides (29). This observation is not consistent with the prevailing model, which suggests that GroEL acts as a post-translational chaperone in *E. coli* (19, 43, 44). This current report describes further investigations of the newly observed behavior of the GroEL system in translation and addresses several questions that arise from our previous observations.

First, we have shown that the stable co-translational interaction of GroEL with the translation complex depends on the length of the nascent polypeptide, using MetK as a model substrate. At present, we cannot exclude the possibility that the C-terminal region of MetK has a specific motif for GroEL binding. Nevertheless, the results here are consistent with previous reports that GroEL has multiple binding sites for denatured proteins (14, 45, 46) and that multivalent binding of substrate

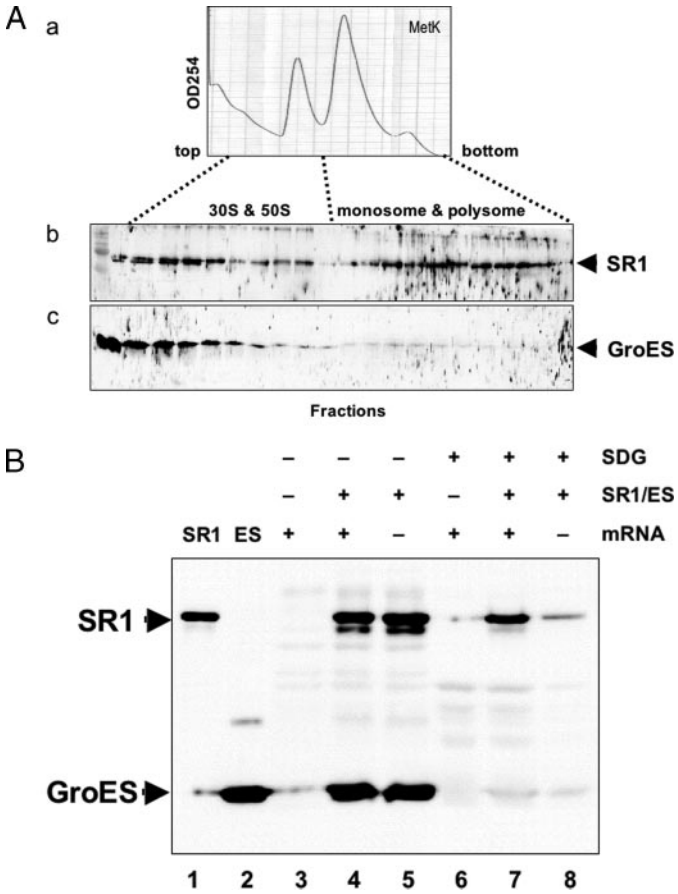

FIGURE 4. **SR1, but not GroES, associates with the translation complexes.** *A, panel a*, gradient fractions from a single-round translation of MetK mRNA after sucrose gradient density centrifugation; *panels b* and *c*, Western blot detection of SR1 and GroES in the fractions. *B*, detection of SR1 and GroES (*SR1 ES*) among the cross-linked translation complexes. Translation complexes were analyzed before (−, *lanes 3–5*) and after (+, *lanes 6–8*) sucrose density gradient (*SDG*) centrifugation. *SR1/ES*, translation in the presence (+, *lanes 3, 4, 7*, and *8*) or absence (−, *lanes 3* and *6*) of SR1 and GroES; *mRNA*, translation in the presence (+, *lanes 3, 4, 6*, and *7*) or absence (−, *lanes 5* and *8*) of the MetK mRNA. *Lanes 1* and *2* are the positive controls for the SR1 and GroES proteins, respectively.

proteins to GroEL is required for folding of stringent substrates (7). In addition, earlier observations that the protein substrates of GroEL *in vivo* are larger than ~10 kDa (8, 10, 11) are also consistent with our results. Further studies on nascent polypeptides other than MetK are necessary to draw a general conclusion that GroEL preferentially associates with longer nascent polypeptides.

The preferential stable binding of GroEL to longer nascent polypeptides might be compatible with the presence of other co-translational chaperones, such as the trigger factor, which can also protect nascent polypeptides from aggregation (24–26). For example, polypeptides emerging from the ribosomal tunnel could associate with the trigger factor, and subsequently, following elongation of the polypeptides, could be exchanged for GroEL. This scheme might cause a transient simultaneous association of both the trigger factor and GroEL with a single nascent chain. Alternatively, GroEL may be required instead of the trigger factor in some cases, as indicated by the recent report showing that the trigger factor was not a general shield for nascent peptides (25). We assumed that such long nascent chains that are not protected by the trigger factor might require GroEL for proper folding. To elucidate the preferential co-translational association between the nascent peptides and individual chaperones, further well designed experiments performed in the presence of the additional trigger factor and/or the DnaK system, besides GroEL, are underway.

It has been estimated that the cellular concentration of GroEL is less than one-tenth that of ribosomes (47, 48), indicating that all nascent polypeptides cannot be protected by the GroEL system. In fact, a proteome-wide analysis of GroEL substrate proteins in GroEL-substrate complexes *in vivo* revealed a set of substrate proteins that stringently need the GroEL system (the class III substrates) (11). What makes substrates stringently dependent on GroEL is not clear, but the nascent chain length-dependent co-translational association of GroEL may provide a rational explanation for the mechanism by which stringent substrates are enriched in the GroEL complexes. Considering that the longer the nascent polypeptides are, the more difficult protein folding is, longer polypeptides that can associate with the multiple binding sites in GroEL may experience a more efficient post-translational assistance by the GroEL system. Furthermore, the formation of such co-translational GroEL binding to the nascent chains has a potential advantage for proteins that stringently require the GroEL system since it may prevent formation of irreversible aggregates.

Secondly, we addressed how GroES assists GroEL-dependent folding of the nascent protein. We showed that both the double-ring GroEL (wild type) and the single-ring SR1 are able to mediate folding of the stringent substrate MetK via GroES capped encapsulation of the substrate. The double-ring GroEL interacted with both the nascent MetK and GroES, whereas SR1 bound to the nascent MetK, but not to GroES, during translation. These results clearly indicate that GroES interacts with the *trans*-ring, but not the *cis*-ring, of the wild-type GroEL associated with the nascent polypeptide. Subsequently, GroES may cap the *cis*-ring of GroEL, which has incorporated the newly synthesized polypeptide detached from ribosome.

Since even SR1 can encapsulate the substrate without the *trans*-GroES interaction, one might ask what are the differences between the double-ring GroEL and SR1 in the encapsulation processes. At least under the condition in which single-round translation was conducted, the efficiencies of the encapsulation processes were similar, indicating that the *trans*-GroES interaction is not necessary for the encapsulation itself. Further, we suggest that the encapsulation by the *cis*-binding of GroES occurs after detachment of the *trans*-bound GroES in the case of the double-ring GroEL.

We previously found an association of GroES with the translation complex in growing *E. coli* (29). Now we can understand that this GroES interaction *in vivo* would be via the *trans*-ring of GroEL, although the *trans*-GroES binding is not a prerequisite for the encapsulation of the substrate polypeptides. Then what is the importance of this *trans*-ring interaction of GroES? We assume that the co-translational binding of the asymmetric GroEL-GroES complex to nascent polypeptides seems to reflect the double-ring GroEL as a "two-stroke engine" (49, 50), resulting in an efficient cycling of GroEL turnover. Since the double-ring GroEL alternates its rings as folding-active *cis*-

complexes (51), the co-translational binding as the asymmetric complex would be appropriate for the efficient use of the limited amount of GroEL in the cell (47, 48). Such a two-stroke mode for GroEL-mediated folding would be extremely important in the cells lacking other co-translational chaperones, trigger factors, or DnaK.

Furthermore, the eukaryotic group II chaperonin CCT/TRiC, which does not require GroES-like co-chaperonin, has been known to work in a co-translational mode (*e.g.* Ref. 52). As CCT/TRiC seems to contain a built-in lid structure (53, 54), the *trans*-ring lid of the nascent peptide-bound CCT/TRiC might be an evolutional consequence of the co-translational *trans*-capping of GroES.

Post-translational capping by GroES would ensure efficient encapsulation of the substrate proteins in the GroEL-GroES cavity. The upper limit of the polypeptides accommodated in the GroEL-GroES cavity is ~57 kDa (17). However, it has been reported that some larger molecular weight proteins serve as substrates for GroEL-GroES, both *in vitro* and *in vivo* (7, 8, 10, 11, 55, 56). The GroEL-polypeptide complex could be capped by GroES exclusively in the *trans*-configuration once these large proteins exceed the encapsulation capacity (55, 56). The co-translational asymmetric complex described here, in which GroES bound to the *trans*-GroEL ring, might represent a complex in which GroES capped the *trans*-ring of GroEL that had bound to a protein too large to be encapsulated. Indeed, the ribosome-nascent polypeptide complex is extremely large when compared with the GroEL cavity. Our results demonstrate that GroEL can contact and associate with the nascent polypeptide portion of this complex as a *holder* chaperone. After that, GroEL may exert a *folder* activity together with the co-chaperonin GroES once the GroEL cavity is spatially sufficient to encapsulate the substrate protein.

In summary, we have revealed that GroEL is not only a post-translational but also a co-translational chaperone in the translation process. An immediate conclusion is that these co- and post-translational roles correspond to the *holder* and *folder* functions, respectively. Thus, the progression from the co- to post-translational function should be guaranteed by the binding of GroES to the *cis*-GroEL ring. In this context, GroES is a key regulator in the chaperonin system for post-translational processes. In eukaryotes, co-translational folding predominates in translation (43, 52, 57, 58). The absence of a direct homolog of GroES in eukaryotes might be an evolutionary consequence of the preference in the folding process.

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