Leu$^{309}$ Plays a Critical Role in the Encapsulation of Substrate Protein into the Internal Cavity of GroEL*

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In the crystal structure of the native GroEL-GroES-substrate protein complex from *Thermus thermophiulus*, one GroEL subunit makes contact with two GroES subunits. One contact is through the H-helices, and the other is through a novel GXXLE region. The side chain of Leu, in the GXXLE region, forms a hydrophobic cluster with residues of the H helix (Shimamura, T., Koike-Takeshita, A., Yokoyama, K., Masui, R., Murai, N., Yoshida, M., Taguchi, H., and Iwata, S. (2004) Structure (Camb.) 12, 1471–1480). Here, we investigated the functional role of Leu in the GXXLE region, using *Escherichia coli* GroEL. The results are as follows: (i) cross-linking between introduced cysteines confirmed that the GXXLE region in the *E. coli* GroEL-GroES complex is also in contact with GroES; (ii) when Leu was replaced by Lys (GroEL(L309K)) or other charged residues, chaperone activity was largely lost; (iii) the GroEL(L309K)/substrate complex failed to bind GroES to produce a stable GroEL(L309K)/GroES-substrate complex, whereas free GroEL(L309K) bound GroES normally; (iv) the GroEL(L309K)/GroES-substrate complex was stabilized with BeF$_2$, but the substrate protein in the complex was readily digested by protease, indicating that it was not properly encapsulated into the internal cavity of the complex. Thus, conformational communication between the two GroES contact sites, the H helix and the GXXLE region (through Leu$^{309}$), appears to play a critical role in encapsulation of the substrate.

Chaperonins are a subclass of molecular chaperones capable of mediating ATP-dependent folding of polypeptides to their native states (1–4). GroEL is the best characterized chaperonin; it is found in the cytoplasm of *Escherichia coli* and is essential for cell viability and growth at all temperatures (5). The complete functional cycle of GroEL is dependent on the presence of ATP and the co-chaperonin GroES (6–11). GroEL is a large cylindrical protein complex comprising two heptamer rings of identical 57-kDa subunits stacked back to back (12). GroES is a dome-shaped, single heptamer ring of 10-kDa subunits (13). GroEL binds a wide variety of substrate proteins in non-native states and forms a binary complex (14–18), which then binds ATP and GroES to the same (cis) GroEL ring to form the cis-ternary complex (8, 9). The binding of GroES induces the encapsulation of the substrate protein into an enlarged cavity (the cis-cavity) inside the cis-ring, which is capped by GroES. In the cis-cavity, non-native protein initiates folding without the risk of aggregation (8, 9, 19). Based on studies of crystal structures and mutagenesis, it is thought that the residues of GroEL involved in binding of GroES are overlapped, to a large extent, with those for binding of the substrate protein (16). Therefore, it might appear that binding of GroES results in freeing of the unfolded protein into the cis-cavity through deprivation of its binding sites. However, simple competition between substrate protein and GroES for the same binding sites does not explain how the release of substrate protein always results in encapsulation into the cis-cavity rather than diffusion into the bulk solution. Analysis of an intermediate in the process of encapsulation may help clarify the mechanism by which GroEL operates at this critical stage.

We recently determined a crystal structure of the native GroEL-GroES complex purified from *Thermus thermophilus*, the cis-cavity of which is filled with cellular proteins (20). The structure shows several significant differences to the GroEL-GroES complex of *E. coli*, which was obtained by reconstitution of purified GroEL and GroES, in the presence of ADP (19). A new contact region between GroEL and GroES was identified in the *T. thermophilus* GroEL-GroES structure (Fig. 1A). In *E. coli* GroES, residues 24–27 are part of a mobile loop structure (comprising residues 24–30) that interacts with helices H and I at the apical domain of GroEL, located in the inner rim of the central cavity. In the GroEL-GroES of *T. thermophilus*, the same interactions are observed. The region Gly$^{305}$, Leu$^{308}$, and Glu$^{309}$ of *T. thermophilus* GroES are well conserved across species, and hereafter, we refer this region as the GXXLE region. This region also has an intrasubunit interaction with the H helix; the side chain of Leu$^{308}$ (Leu$^{309}$ in *E. coli* GroEL) points at the N terminus of the H helix to form a hydrophobic cluster with other residues (Fig. 1, A, circle, and B). Fenton et al. (16) reported that a Leu$^{309}$ mutant of *E. coli* GroEL (GroEL(L309K)) was unable to assist folding. The aim of this study was to examine the contribution of the GXXLE region to chaperonin function, using *E. coli* GroEL and GroES. We investigated the GXXLE region in the *E. coli* GroEL-GroES complex to determine whether it is also in contact with GroES and whether or not Leu$^{309}$ in *E. coli* GroEL plays a role for the efficient encapsulation of substrate protein into the cis-cavity.

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### EXPERIMENTAL PROCEDURES

**Reagents and Proteins**—BeCl₂ was from Aldrich. NaF was obtained from Wako (Osaka, Japan). Chymotrypsin and hexokinase were from Sigma. Porcine malate dehydrogenase (MDH), ATP, and ADP were obtained from Roche. The trace amount of contaminating ATP in the ADP solution was eliminated by hexokinase/glucose treatment (21). Cy3-NHS (Fluorolink Cy3 monofunctional dye) was from Amersham Biosciences. The following proteins were purified and prepared as previously described: green fluorescent protein (GFP) (22); GroEL, GroES, and bovine mitochondrial rhodanese (23); and Cy3-labeled GroES (GroESCy3) and Cy3-labeled MDH (MDHCy3) (24).

**Strains and Plasmids**—E. coli XL-2 Blue (Stratagene) was used for site-directed mutagenesis and cloning. E. coli GroEL mutants were generated using QuikChange site-directed mutagenesis (Stratagene). The mutated groEL gene fragment was amplified using PCR, and the mutation containing pET-EL plasmid was used as a template. PCR products were digested with NcoI and HindIII and ligated into the NcoI/HindIII site of pTV118N (Takara), forming pTV-EL. The wild type groEL gene fragment was amplified using PCR, digested with Sall and EcoRI, and ligated into the Sall/EcoRI site of pSTV29, forming pSTV-ES. E. coli MM100 (supplied by Dr. M. Masters) was used for complementation experiments (25). Mutated GroEL and wild type GroES were co-expressed (from expression plasmids pTV-EL and pSTV-ES, respectively) in E. coli MM100.

**Formation of the GroEL Cross-linked Product**—The mixtures (40 µl) containing HKM buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, and 5 mM MgCl₂), 0.25 µM GroEL, 0.5 µM GroES, 1 mM dithiothreitol (DTT) and, when indicated, 1 mM ATP, were loaded onto centrifugal ultrafiltration units (Microcon YM-10) to remove DTT gradually. After treatment for 60 min, 200 µM iodoacetamide was added to a final concentration of 10 µM to prevent excessive cross-linking. The intramolecular cross-linked product was formed as follows: 40 µl of mixture containing HKM buffer, 0.25 µM GroEL (E232C/L309C), 20 µM CuCl₂ and, when indicated, 1 mM ATP, 1 mM ADP, 1 mM DTT, and 0.5 µM GroES was incubated at 25 °C. After 30 min, iodoacetamide was added to a final concentration of 10 µM. Cross-linked products (~10 µg) were analyzed by polyacrylamide gel electrophoresis using 0.1% SDS (SDS-PAGE) in the absence of reducing agent.

**Folding Assays**—For measurement of GFP folding, an acid-denatured GFP solution (12.6 µM) was diluted 252-fold in HKM buffer (1.3 ml) containing 0.15 µM GroEL, 5 mM DTT, 200 mM glucose, and 0.3 µM GroES. Where indicated as “+ trap”, this was followed by the addition of 0.04 unit/µl hexokinase and 0.3 µM “trap-GroEL” (GroEL(N265A) (8)); trap-GroEL binds free unfolded proteins irreversibly even in the presence of ATP. Then 0.8 mM ATP was added to initiate folding. The intensity of GFP fluorescence was monitored continuously with a fluorometer (excitation at 485 nm, emission at 510 nm; FP-6500, Jasco). MDH was denatured in 6 M urea and 1 mM DTT for 1 h and diluted in HKM buffer containing 0.1 µM GroEL, 0.3 µM GroES, 5 mM DTT, and 2 mM ATP. The final MDH subunit concentration was 0.2 µM. At the times indicated, a 25-µl aliquot was injected into 1.2 ml of the assay solution containing 0.5 mM oxalacetic acid, 0.2 mM NADH, 1 mM DTT, and 0.1 mg/ml bovine serum albumin. The rate of oxidation of NADH at 25 °C was monitored at 340 nm. Rhodanese was denatured in 6 M guanidine HCl (20 mM) and 1 mM DTT for 1 h and diluted 40-fold into HKM buffer containing 1 µM GroEL, 2 µM GroES, 20 mM Na₂S₂O₃, and 1 mM DTT. ATP was then added to a final concentration of 4 mM. At the times indicated, 5-µl aliquots were added to 750 µl of a solution containing 100 mM KH₂PO₄, 150 mM Na₂S₂O₃, and 1 mM EDTA. Recovery of rhodanese activity was measured colorimetrically by absorbance at 460 nm, indicating formation of a complex between ferric ions and the thio-cyanate reaction product (26).

**Binding Assays Using Gel Filtration**—The GroEL-Cy3-labeled MDH (MDHCy3) complex was formed in the presence or absence of GroES, and the GroEL-Cy3-labeled GroES (GroESCy3) complex was formed in the presence or absence of denatured MDH. Denatured MDH (or MDHCy3) was diluted in HKM buffer containing GroEL and incubated for 2 min at 25 °C. The solution containing ATP, with (or without) GroES, was added and incubated for 2 min at 25 °C. Final concentrations of the components were 0.125 µM MDH (or MDHCy3), 0.25 µM GroEL, 0.125 µM GroES, and 0.5 mM ATP. Aliquots (100 µl) were loaded onto a gel filtration HPLC column (G3000SW_XL; Tosoh, Japan) equilibrated with HKM buffer containing 50 mM Na₂SO₄ and 0.2 mM HEPES-KOH, pH 7.4, 100 mM KCl, and 5 mM MgCl₂. GroEL and GroES were incubated at 25 °C. After 30 min, iodoacetamide was added to a final concentration of 10 µM. Cross-linked products (~10 µg) were analyzed by polyacrylamide gel electrophoresis using 0.1% SDS (SDS-PAGE) in the absence of reducing agent.
**Novel Contacts between GroEL and GroES**

**Results**

**Conservation of Novel Contacts in the *E. coli* GroEL-GroES Complex**—To investigate whether or not the GXXLE region is in contact with GroES in the *E. coli* GroEL-GroES complex, we conducted a series of cross-linking experiments using mutants of *E. coli* GroEL and GroES. We replaced Met307, Glu308, Leu309, and Glu310 in GroEL, and Thr28, Gly29, and Ser30 in GroES with Cys. The removal of DTT from the reaction mixture containing GroEL and GroES led to the generation of a single high molecular mass band, corresponding to cross-linked GroEL-GroES (Fig. 2A and B). Binding of GroES to GroEL is known to be ATP (or ADP)-dependent, and the cross-linking was only successful when ATP was present. Among the mutants, the combination GroEL(E308C)/GroES(S30C) was most efficiently cross-linked, followed by the combination of GroEL(E308C)/GroES(G29C). We found efficient intrasubunit cross-linking between L309C and E232C at the N terminus of the H helix (Fig. 2C), and cross-link formation was not affected by nucleotides or GroES. These results confirm that the topological arrangement of the GXXLE region in the GroEL-GroES complex of *E. coli* is similar to that of the *T. thermophilus* complex, where the mobile loop of GroES and the H helix interact with the GXXLE region.

**Effect of Mutation of Leu309 on Growth of *E. coli***—The results above indicate that Glu308 of GroEL and Gly29/Ser30 of GroES are in close proximity. Next we investigated a role for the highly conserved Leu309 in chaperonin function. We replaced Leu309 of GroEL with Val, Ala, Asn, Asp, and Lys. *E. coli* MM100 (25), a strain in which expression of chaperomosal GroEL-GroES is under control of the *P_BAD* promoter (arabinose induction), was co-transformed with the expression plasmids encoding the GroEL Leu309 mutants and wild type GroES. Transformants were cultured on LB plates, in the absence of arabinose (Fig. 3). Cells with expression plasmids encoding the mutants GroEL(L309A) and GroEL(L309V) grew normally, as did cells expressing wild type GroEL. In contrast, as previously described by Fenton et al. (16), cells expressing the mutant GroEL(L309K) could not grow in the absence of arabinose. Similarly, cells expressing the mutants GroEL(L309N) or GroEL(L309D) were unable to rescue GroEL-deficient *E. coli* MM100. Thus, the GroEL mutants, in which Leu309 was replaced by polar residues, could not support growth of GroEL-deficient *E. coli* MM100, indicating a critical role for Leu309 in chaperonin function *in vivo*.

**Chaperone Activity of the Leu309 Mutants**—The GroEL Leu309 mutants were purified, and their properties were examined. ATPase ATP. The flow rate was 0.5 ml/min, and elution was monitored by an in-line fluorometer (excitation at 550 nm, emission at 570 nm).

**Protease Sensitivity of Substrate Protein in the GroEL-GroES Complex**—GroEL that had been saturated with denatured rhodanese was prepared as described previously (21, 27). The reaction mixtures contained 1 mM nucleotide, 10 mM NaF, 2 mM BeCl2, 20 mM Na2S2O3, 1 μM rhodanese-saturated GroEL, 2.0 μM GroES, and 1 mM DTT in HKM buffer. Unbound GroES and substrate proteins were removed by ultrafiltration (Microcon YM-100) at 90 min after initiation of the reaction. Chymotrypsin (final concentration, 1 μg/ml) and glycerol (final concentration, 10% v/v) were added to 25 μl of the mixture containing 1 mM DTT and 15 μg of protein in HKM buffer. Following incubation for 20 min at 25 °C, components with a molecular mass of <100 kDa were removed by ultrafiltration (Microcon YM-100). An aliquot of the resulting solution was analyzed by 13% SDS-PAGE. The intensity of band staining was quantified using the NIH Image program and calibrated using known protein concentrations.

**Figure 2. Cross-linking between cysteine-incorporated *E. coli* mutants of GroEL and GroES.** A and B, intermolecular cross-linking between mutants of GroEL and GroES. The mixture containing purified *E. coli* GroEL and GroES mutants with 1 mM DTT was subjected to ultrafiltration to remove DTT gradually and in the absence (A) or presence (B) of 1 mM ATP to induce cross-linking. C, intrasubunit cross-linking between the GXXLE region and the H helix in GroEL. The GroEL(E232C/L309C) double cysteine mutant was incubated with 20 μM CuCl2 in the absence or presence of GroES and 1 mM nucleotides. DTT was included in the leftmost sample (lane 1). Products were analyzed by SDS-PAGE in the absence of a reducing agent, and gels were stained with Coomassie Blue.

**Figure 3. Complementation of *E. coli* MM100 by expression of mutant GroEL proteins.** The viability of *E. coli* MM100 cells co-expressing GroES and either wild type (WT) or mutant GroEL when plated on LB in the absence of arabinose is shown.
activities of the mutants were similar to those of the wild type (data not shown). Protein folding activity was tested using GFP. Denatured GFP was diluted into a solution of GroEL and GroES. Upon dilution, denatured GFP was bound efficiently to the GroEL mutant, because no spontaneous GFP folding occurred (Fig. 4A, inset). GFP started folding upon the addition of ATP, and regardless of mutations, a similar yield of folded protein (~70%) was achieved after 200 s (Fig. 4A). In the parallel experiments, hexokinase was included in the mixtures to eliminate ATP and to prevent the secondary turnover of the GroEL reaction cycle. Excess trap-GroEL (GroEL(N265A)) (8) was added prior to ATP addition, to capture unfolded proteins in the bulk solution. Under these conditions, only folding of proteins encapsulated in the cis-cavity during the first round of the GroEL reaction cycle would be observed. Under the single cycle reaction conditions, the yield of folded GFP differed among the mutants (Fig. 4B). Two mutants, GroEL(L309A) and GroEL(L309V), retained wild type-like folding activity, whereas GroEL(L309N), GroEL(L309D), and GroEL(L309K) gave significantly reduced yields of folded protein. These results indicate that the GroEL mutants in which Leu309 was replaced by polar residues tend to fail in encapsulating unfolded proteins into the cis-cavity. In addition, we tested the effect of these mutations on the folding of stringent substrate proteins, such as MDH and rhodanese, the folding of which depends on the GroEL/GroES system. GroEL(L309V) and GroEL(L309A) mediated efficient folding of both proteins, with the former giving a better yield of folded protein than wild type GroEL (Fig. 4C). As expected, substitution of Leu309 by polar residues, particularly Lys and Asp substitutions, resulted in a significant decrease in the yields of reactivated proteins (MDH and rhodanese).

Binding of Substrate Protein and GroES to GroEL(L309K)—In the light of previous investigations of GroEL(L309K) by Fenton et al. (16), we sought to analyze further the properties of GroEL(L309K). The mutant protein was mixed with denatured MDHCy3 and analyzed using gel filtration HPLC with elution in a buffer containing ATP (Fig. 5A). Like the wild type protein, GroEL(L309K) bound and retained MDHCy3. The binding of MDHCy3 to GroEL(L309K) was not affected by the presence of GroES in the mixture (Fig. 5B). Next we investigated the binding of GroEScy3. In the absence of denatured MDH, GroEL(L309K) formed a complex with GroEScy3 (Fig. 5C). However, in the presence of denatured MDH, GroEScy3 was primarily eluted as the free GroES heptamer (Fig. 5D). Under the same conditions, the wild type GroEL formed a complex with both MDHCy3 (Fig. 5B) and GroEScy3 (Fig. 5D). These results demonstrate that GroEL(L309K) can bind denatured substrate protein or GroES individually but cannot form a GroEL-GroES-MDH cis-ternary complex that has sufficient stability to survive gel filtration. cis-Ternary Complexes of GroEL(L309K) Formed in BeFx and Their Sensitivity to Protease Digestion—In general, BeFx can mimic the phosphate of enzyme-bound nucleotides and stabilize transient complexes in ATP- and GTP-metabolizing proteins (28). In the case of GroEL, BeFx stabilizes the cis-ternary complex of GroEL; a 1:2:2 GroEL-GroES-substrate protein complex with double cis-cavities and a 1:1:2 GroEL-GroES-substrate protein complex with a single cis-cavity are formed with ATP and ADP, respectively (27). Each cavity contains a substrate protein that is able to fold and that is protected from the attack by protease (27). In anticipation that BeFx would also stabilize the cis-ternary complex of GroEL(L309K), we included BeFx in the reaction mixtures that contained rhodanese as a substrate protein. The complex was isolated using ultrafiltration and analyzed with SDS-PAGE (Fig. 6, lanes 1–6). In a control experiment, 1 mol of free wild type GroEL or GroEL(L309K) (without GroES, nucleotide and BeFx) bound 2 mol of rhodanese (lanes 1 and 2). The GroEL(L309K) complex that formed in
the presence of ATP and BeF₄, GroEL(L309K)-GroESrhodanese, had an apparent composition of 1:2:1 (lane 4). This indicated that, for the GroEL(L309K) mutant, one of the two rhodanese molecules in the 1:2:2 GroEL-GroESrhodanese complex (lane 3) had dissociated. The GroEL(L309K) complex, formed in the presence of ADP and BeF₄, had an apparent composition of 1:1:2 (GroEL(L309K)-GroESrhodanese) (lane 6). This composition is the same as found for wild type (lane 5). In this complex, one substrate occupies the cis-cavity, whereas the other is positioned on the opposite (trans) GroEL ring, without GroES. In parallel experiments, the isolated complexes were treated with chymotrypsin, reisolated using ultrafiltration, and analyzed with SDS-PAGE (Fig. 6). The rhodanese molecules that had bound to free GroEL (wild type or GroEL(L309K)) were digested completely (lanes 7 and 8). Rhodanese molecules in the 1:2:2 GroEL-GroESrhodanese complex were fully protected from digestion (lane 9). Rhodanese molecules in

FIGURE 6. Sensitivity of substrate protein trapped in the GroEL complexes to protease digestion. GroES, ATP, and BeF₄, or GroES, ADP, and BeF₄, were added to GroEL (for both wild type (WT) and the L309K mutant) that had been saturated with denatured rhodanese. After 90 min, the aliquots underwent one of the two following treatments: ultrafiltration (100-kDa cut) and SDS-PAGE (lanes 1–6); or ultrafiltration (100-kDa cut), chymotrypsin treatment, a second ultrafiltration (100-kDa cut), and SDS-PAGE (lanes 7–12). The gels were stained with Coomassie Blue.

the 1:2:1 GroEL(L309K)-GroESrhodanese complex were digested completely (lane 10). For the complexes formed in the presence of ADP and BeF₄, only one rhodanese molecule in the wild type 1:1:2 GroEL-GroESrhodanese complex was digested (lane 11). Both rhodanese molecules in the 1:1:2 GroEL(L309K)-GroESrhodanese complex were digested (lane 12). Thus, the substrate proteins in the GroEL(L309K)-GroES complexes are all accessible to attack by chymotrypsin. Similar results were obtained when MDH was used as a substrate protein (data not shown). It appears, therefore, that GroEL(L309K) can form a relatively stable ternary complex with GroES and substrate proteins in the presence of BeF₄; however, it cannot prop-

FIGURE 7. Cross-linking between GroES(S30C) and GroEL(E308C) in the GroEL(L309K) mutant. Cross-linking was performed between GroES(S30C) and GroEL(E308C) (lanes 1 and 2) or GroES(S30C) and GroEL(E308C/E309K), in which a second mutation, GroEL(E308C), was introduced into GroEL(L309K) (lanes 3 and 4). The mixture containing 1 mM DTT and the two mutants was subjected to ultrafiltration as described in the legend to Fig. 2 in the presence of either ATP (lanes 1 and 3) or ATP and BeF₄ (lanes 2 and 4).
Novel Contacts between GroEL and GroES

Leu<sup>109</sup> Plays a Critical Role in the Encapsulation of Substrate—It was previously reported by Fenton et al. (16), that GroEL(L309K) was unable to associate with either folding in vitro or the rescue of GroEL-deficient E. coli. The results from this study indicate that GroEL(L309K) cannot properly encapsulate substrate protein into the cis-cavity. It appears that the GroEL(L309K)-substrate protein complex cannot bind GroES in a stable and productive manner. When the complex is stabilized by BeF<sub>4</sub>, the substrate protein is unable to fold and is susceptible to protease digestion. This indicates that the substrate protein in the BeF<sub>4</sub>-stabilized complex is still tethered to GroEL and is exposed to the outer side medium because of incomplete cupping by GroES. It is possible that GroES cannot completely sequester the common binding sites on all GroEL subunits, and so both GroES and the substrate protein remain bound to GroEL. Because BeF<sub>4</sub> is thought to stabilize transient complexes in many ATP-metabolizing proteins, it is likely that the wild type GroEL also forms a transient intermediate like the GroEL(L309K)-GroES-substrate protein complex, before assuming the productive cis-ternary complex structure. If this suggestion is correct, then the next step of the wild type GroEL reaction cycle must be the discharge of substrate into the cis-cavity, accompanied by completion of GroES capping. The contention above is similar to the two-tier model of GroEL functioning (11). In that model, the GroEL-substrate complex binds GroES to generate a key intermediate whereby the substrate protein is tethered at some point to GroEL and, of this complex (~3 s) accompanies discharge of the substrate protein into the cis-cavity. In our experiments, the substrate protein was still in a non-native, protease-susceptible state in the BeF<sub>4</sub>-stabilized GroEL(L309K)-GroES-substrate complex. Thus, this ternary complex may resemble the intermediate described in the two-tier model.

The Role of the GXXLE Region—GroEL(L309K) and the other mutants in the GXXLE region bind substrate protein normally (Figs. 4 and 5, A and B). In nucleotide-dependent GroES binding, GroEL(L309K) binds GroES normally in the absence of substrate (Fig. 5C). This demonstrates that the GXXLE region does not contribute to GroES binding in the absence of substrate. These results confirm previous studies showing that the H-I helices of GroEL are sufficient for GroES binding (16, 19). In contrast, the GroEL(L309K)-substrate protein complex binds GroES poorly (Fig. 5D), unless BeF<sub>4</sub> is included in the reaction mix to stabilize the transient ATP-bound complex (Fig. 6). These findings indicate that the integrity of the GXXLE region is critical for the binding of GroES to the GroEL/substrate protein complex. The following model suggests a means by which Leu<sup>109</sup> plays a pivotal role in subunit association and function (Fig. 1B). When associated with GroEL, one GroES subunit interacts with two adjacent GroEL subunits, using different stretches of the mobile loop (Fig. 1B). Similarly, one GroEL subunit is in contact with two adjacent GroES subunits at the H-I helices and the GXXLE region (Fig. 1B). The two contact sites in a GroEL subunit can communicate with each other through a hydrophobic cluster formed by the side chain of Leu in the GXXLE region and residues at the entrance formed by the H helix. Consequently, in the whole GroEL-GroES ring structure, these contacts form an interaction sequence connecting all of the GroES/substrate-binding sites in the apical domain of GroEL and the mobile loop of GroES. Therefore, one can assume that binding of one GroES subunit to the GXXLE region can induce conformational transitions in the H helix, which can stimulate release of the substrate and free the binding site for the next GroES subunit. If communication between the GXXLE region and the H helix is interrupted, as in the L309K mutation, the interaction sequence will halt and produce an unstable ternary complex. However, further study is required to confirm this hypothesis.

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