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Escherichia coli DnaK and GrpE Heat Shock Proteins Interact Both In Vivo and In Vitro

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Previous studies have demonstrated that the *Escherichia coli dnaK* and *grpE* genes code for heat shock proteins. Both the DnaK and GrpE proteins are necessary for bacteriophage λ DNA replication and for *E. coli* growth at all temperatures. Through a series of genetic and biochemical experiments, we have shown that these heat shock proteins functionally interact both in vivo and in vitro. The genetic evidence is based on the isolation of mutations in the *dnaK* gene, such as *dnaK9* and *dnaK90*, which suppress the Tr^- phenotype of bacteria carrying the *grpE280* mutation. Coimmunoprecipitation of $DnaK^+$ and $GrpE^+$ proteins from cell lysates with anti-DnaK antibodies demonstrated their interaction in vitro. In addition, the DnaK756 and GrpE280 mutant proteins did not coimmunoprecipitate efficiently with the $GrpE^+$ and $DnaK^+$ proteins, respectively, suggesting that interaction between the DnaK and GrpE proteins is necessary for *E. coli* growth, at least at temperatures above 43°C. Using this assay, we found that one of the *dnaK* suppressor mutations, *dnaK9*, reinstated a protein-protein interaction between the suppressor DnaK9 and GrpE280 proteins.

After being subjected to a sudden rise in temperature, all organisms tested, from *Escherichia coli* to *Homo sapiens*, induce the synthesis of a group of so-called heat shock (HS) proteins, some of which are highly conserved among species (for reviews, see references 9, 21, and 25). A number of laboratories have begun to investigate the properties of these proteins and their expression. Regulation of the HS response is understood in part. In *E. coli*, induction of the HS response occurs at the transcriptional level under the control of the *rpoH* gene product, σ^{32}. The σ^{32} polypeptide allows RNA polymerase enzyme to specifically recognize HS promoters which have a consensus sequence distinct from that recognized by the normal sigma factor, σ^{70}, encoded by *rpoD* (8, 15). One of the negative regulators of the HS response is the DnaK HS protein (30). This 70-kilodalton protein shows approximately 50% amino acid identity with the Hsp70 family of proteins present in all cells examined (4, 9, 21). DnaK and its eucaryotic homologs appear to mediate protein stabilization, refolding, and dissociation (6, 19, 26). As an example, during initiation of λ DNA replication, the DnaK and DnaJ HS proteins, which are essential for λ DNA replication, catalyze the ATP-dependent dissociation of λ P protein from a complex which includes the λ O, λ P, and *E. coli* DnaB proteins assembled at *ori*λ (20). The λ P protein binds to DnaB and inhibits its helicase activity, which is absolutely necessary for the initiation of λ DNA replication (20).

The *E. coli* $GrpE^+$ HS protein is of interest because it forms a complex with the $DnaK^+$ protein. It binds specifically to a $DnaK^+$ affinity column in a salt-resistant manner and is released in the presence of ATP (36). Intracellular ATP levels appear to fluctuate during the HS response (18), and this fluctuation may be necessary for the induction of thermotolerance (17). The concentration of Hsp70 protein in cells is generally thought to correlate with the degree of thermotolerance (9, 21). Potentially, the $GrpE^+$ protein modulates DnaK activity during the HS response in a manner that is sensitive to the ATP concentration in the cell.

Here we present evidence that the *E. coli* DnaK and GrpE proteins interact in vivo. We determined this by isolating extragenic suppressors of the *grpE280* mutation which confer bacterial temperature resistance (Tr^+) and which map in the *dnaK* gene. Coimmunoprecipitation of both proteins from cell extracts with polyclonal antibodies against DnaK protein indicated that the interaction of DnaK with GrpE was quite stable even under harsh experimental conditions. We report that coprecipitation of the mutant DnaK756 or GrpE280 proteins with $GrpE^+$ or $DnaK^+$, respectively, was greatly reduced, suggesting a correlation between the inability of the mutant proteins to interact and the temperature sensitivity (Tr^-) phenotype of these strains. Furthermore, the *dnaK9* mutation, which restored the ability of *E. coli grpE280* to grow at high temperature, also restored the ability of DnaK9 protein to bind to GrpE280 protein. These results suggest that binding between DnaK and $GrpE^+$ proteins may be important for both λ DNA replication and cell viability at high temperature.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this work are shown in Table 1.

Media. L broth contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. The pH was adjusted to 7.4 by adding NaOH. T broth was L broth without yeast extract. T-agar and L-agar plates were prepared from the corresponding broths by adding 10 g of agar (Difco Laboratories) per liter. For selection of Kan^r or Amp^r, the media were supplemented with the corresponding antibiotic to a final concentration of 50 μg/ml. The composition of high-sulfur M9 medium for labeling with [^{35}S]methionine has been described previously (12, 14).

P1 transduction experiments. Phage P1L4 was grown and used in transduction experiments as described by Miller (24).

Electrophoresis. One-dimensional gel electrophoresis was carried out as described previously (12, 14). Sodium dodecyl sulfate (SDS)-polyacrylamide (12.5%) was used.

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TABLE 1. Bacteria, plasmids, and bacteriophages used

Strain, plasmid, or phage	Genotype or phenotype	Source (reference)
Strains		
DA15	W3101 *grpE*$^+$ *pheA*::Tn*10*	D. Ang (2)
DA16	W3101 *grpE280 pheA*::Tn*10*	D. Ang (2)
CG25	W3101 *sup*^{0}	13
CJ1	DA16 *zac*::mini-Tn*10* Kan^r; 90–95% cotransducible with *dnaK*$^+$	This work
CJ2	CJ1 *dnaK9*	This work
CJ3	CJ1 *dnaK90*	This work
CJ4	CG25 *dnaK9 zac*::mini-Tn*10* Kan^r; 90–95% cotransducible with *dnaK9*	This work
CJ5	CG25 *dnaK90 zac*::mini-Tn*10* Kan^r; 90–95% cotransducible with *dnaK90*	This work
CG333	*dnaK103*; no colony formation at 42°C	K. von Meyenburg
Plasmids		
pBR322	Multicopy; Amp^r Tet^r	5
pCG1	pBR322 carrying 5.4-kb[a] *E. coli dnaK*$^+$ *Hin*dIII fragment	This work
pCG2	pCG1 with Ω cassette (Spc^r) in unique *Eco*RI site of *dnaK*; *dnaK* mutant	This work
pJK23	pEMBL8$^+$ *grpE*$^+$ Amp^r	22
Bacteriophages		
P1	P1L4; clear plaque former	Lucien Caro
λ L47.1	λ cloning vector	23
λ *grpE*$^+$	λ L47.1 *grpE*$^+$	This work
λ *dnaB*$^+$	λ L47.1 *dnaB*$^+$	This work
λ *dnaJ*$^+$	λ L47.1 *dnaJ*$^+$	This work
λ *dnaK*$^+$	λ L47.1 *dnaK*$^+$	This work
λNK1105	λ *imm*$^\lambda$ Pam80 mini-Tn*10* Kan^r; ptac-Tn*10* transposase	33
λ *imm*^{21} *dnaK*$^+$	*cI*$^+$ *att*$^{\lambda+}$ *int*$^+$ *dnaK*$^+$	11
λ *imm*^{21} *dnaK*::Ω	*cI*$^+$ *att*$^{\lambda+}$ *int*$^+$ *dnaK*::Ω (Spc^r); Ω insertion inactivates DnaK	This work

[a] kb, Kilobases.

Selection of a kanamycin resistance marker near *dnaK*. Random mini-Tn*10*Δ16Δ17 Kan^r insertions into the bacterial chromosome were obtained by using λ NK1105 as described elsewhere (33). Selection of a mini-Tn*10* insertion near *dnaK* was done by simultaneously transducing an *E. coli dnaK103* strain to Kan^r and Tr$^+$ at 43°C. In subsequent experiments, it was shown that the mini-Tn*10* Kan^r insertion was 90 to 95% cotransducible with the *dnaK* gene.

Plasmid transformation. Mutant *E. coli dnaK9* and *dnaK90* bacteria were grown in L broth at 37°C to a density of approximately 2×10^8 cells per milliliter. Two milliliters of culture was pelleted at 10,000 × *g* for 5 min and washed with 20 mM $CaCl_2$. The cells were suspended in 200 μl of 50 mM $CaCl_2$, plasmid DNA was added, and the suspension was kept on ice for 1 to 12 h. The tubes were kept at 42°C for 2 min and then placed on ice for 15 min. One milliliter of L broth was added to each tube; then the tubes were incubated at 30°C for 1 h to allow expression of ampicillin resistance. A 100-μl portion of this culture was spread on an L-broth plate with ampicillin and incubated overnight at 30°C.

Labeling experiments. Bacteria were grown in high-sulfur M9 medium at 30°C to a density of 4×10^8 cells per milliliter. Samples were removed and transferred to prewarmed flasks. The cultures were incubated at 43°C with 20 μCi of Trans^{35}S-Label (ICN Pharmaceuticals Inc.) per ml. This product contains 70% L-[^{35}S]methionine and 15% L-[^{35}S]cysteine. After a 20-min labeling period, the labeling reaction was stopped by adding excess nonradioactive methionine and cysteine. All the following steps were performed at 0 to 4°C. Bacteria were pelleted at 15,000 × *g* for 5 min at 4°C. The pellets were washed and suspended in M9 medium. Lysis and DNase-RNase treatments were performed essentially as described in reference 35. The lysates were centrifuged at 30,000 × *g* in a Beckman JA-20 rotor for 25 min at 4°C. The supernatants were stored at −70°C.

Preparation of DnaK and GrpE polyclonal antibodies. The DnaK protein was purified as previously described (36; S. Sell, Ph.D. dissertation, University of Utah, Salt Lake City, 1987) by a procedure devised for the purification of Hsp70 from HeLa cells (34). Anti-DnaK antibodies were prepared as described elsewhere (37). The GrpE$^+$ protein was purified by using a procedure described previously (36). Protein (500 μg) was emulsified with Freund complete adjuvant and injected subcutaneously into the backs of 1-year-old female New Zealand White rabbits.

Immunoprecipitations. Extracts from approximately 1.3×10^8 [^{35}S]methionine-labeled cells were incubated with 5 μl of anti-DnaK serum and kept on ice for 3 h. An equal volume of a 10% *Staphylococcus aureus* Cowan cell solution (Boehringer Mannheim Biochemicals) was added, and incubation was continued on ice for an additional 3 h. The precipitate was pelleted at 15,000 × *g* at 4°C, washed three times in RIPA buffer (25 mM Tris, pH 7.5–1% Triton X-100–1% deoxycholate–0.1% SDS–150 mM NaCl) and boiled in SDS loading buffer (12, 14). The samples were pelleted (15,000 × *g*) for 3 min, and the supernatant was loaded immediately onto a 12.5% SDS-polyacrylamide gel. The precipitation was carried out overnight on ice with the same amount of antibody as used before to determine whether washing with RIPA buffer reduces the amount of GrpE protein precipitated with anti-DnaK polyclonal antibody. The precipitation solutions were pelleted at 37,000 × *g* for 1 h at 5°C. The pellet was washed three times with 50 mM Tris (pH 7.5)–20% (vol/vol) glycerol and once with the Tris buffer alone and was then suspended in 10 μl of H_2O. SDS loading buffer was added, and the mixture was electrophoresed on a 12.5% SDS-polyacrylamide gel. The two methods gave identical results for GrpE coprecipitation with DnaK protein.

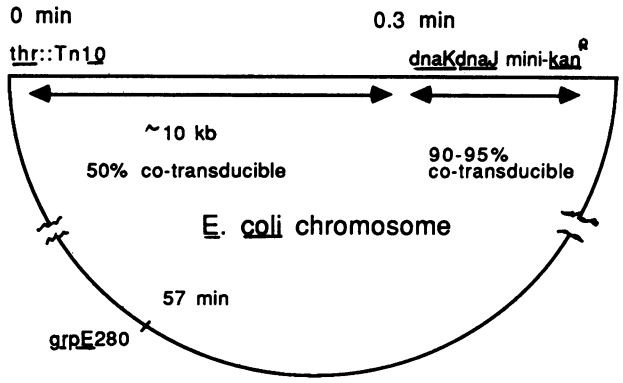

FIG. 1. Relative positions of the various genetic elements used in this work. The cotransduction frequencies shown were obtained with phage P1L4 (2). kb, Kilobases.

RESULTS

Isolation of second site suppressors of *grpE280* in the *dnaK* region of the *E. coli* genome. The *E. coli grpE280* mutant strain originally was isolated by its ability to block replication of the λ *N*am7am53 *c*Itsl-2 prophage at 42°C (28). Upon being tested, this strain was found to be unable to form colonies at 43.5°C (2). Subsequent transduction and reversion analyses demonstrated that the Tr^- phenotype is due to the *grpE280* mutation (2). Our aim was to isolate mutations which suppress the GrpE280 Tr^- phenotype and simultaneously map in the *dnaK* gene. To achieve this goal, we introduced into *grpE280* bacteria a mini-Tn*10* marker (33) carrying resistance to kanamycin and closely linked to *dnaK* (90 to 95% by P1 transduction [Fig. 1]). This strain was incubated on L-agar plates at 44°C for 24 to 48 h. Temperature-resistant (Tr^+) survivors were obtained at a frequency of approximately 10^{-7}.

Theoretically, such revertants should fall into three classes: (i) true revertants, (ii) intragenic suppressors, and (iii) extragenic suppressors. Since we were interested only in extragenic suppressors, we screened for this type of mutation by testing the Tr^+ revertants for the ability to propagate λ *grpE*$^+$ transducing phage. The λ *grpE*$^+$-resistant colonies were expected to be due chiefly to extragenic mutations which result in a block to λ growth or, potentially, to *grpE trans*-dominant mutations. We found that approximately 10% of the *grpE280* Tr^+ revertants were simultaneously resistant to the λ *grpE*$^+$ transducing phage. The majority of these λ *grpE*$^+$-resistant suppressors mapped in or near the *dnaK* gene, as shown by the following transduction experiments. Bacteriophage P1 lysates were grown on these Tr^+ colonies and used to transduce *E. coli grpE280* bacteria to Kan^r at 30°C. Colonies from each transduction were subsequently tested for growth at both 30 and 44°C. The colonies that grew at 44°C were chosen as candidates for suppressors of *grpE280* which map in the *dnaK* gene.

Phenotype of *grpE280* suppressors in a wild-type background. Two of the candidate suppressor mutations (*dnaK9* and *dnaK90*), which were 90 to 95% linked to mini-Tn*10* Kan^r near the *dnaK* gene, were the subjects of further experimentation. The *dnaK9* and *dnaK90* mutations were transduced into the CG25 wild-type background by means of P1 transduction, selecting for Kan^r. These bacteria were then checked for λ plating characteristics in an attempt to find an identifiable phenotype. The results are shown in Table 2.

TABLE 2. Plating properties of phages λ L47.1 and λ L47.1 *grpE*$^+$ on different bacterial strains[a]

Bacterial strain	Plating property[b]	
	λ L47.1	λ L47.1 *grpE*$^+$
dnaK$^+$ *grpE*$^+$	+	+
dnaK$^+$ *grpE280*	−	+
dnaK9 grpE280	−	−
dnaK9 grpE$^+$	±	−
dnaK9 grpE$^+$(pBR322*dnaK*$^+$)	+	+
dnaK9 grpE$^+$(pBR322*dnaK*::Ω)	±	−
dnaK9 grpE$^+$(λ *imm*^{21} *dnaK*$^+$)	±	−
dnaK9 grpE$^+$(λ *imm*^{21} *dnaK*::Ω)	±	−
dnaK90 grpE280	+	−
dnaK90 grpE$^+$	±	−
dnaK90 grpE$^+$(pBR322*dnaK*$^+$)	+	+
dnaK90 grpE$^+$(pBR322*dnaK*::Ω)	±	−
dnaK90 grpE$^+$(λ *imm*^{21} *dnaK*$^+$)	+	+
dnaK90 grpE$^+$(λ *imm*^{21} *dnaK*::Ω)	±	−

[a] The incubations were carried out at 37°C.

[b] +, Good phage growth, with an efficiency of plating of 0.5 to 1.0; ±, reduced phage growth, with smaller plaque size and an efficiency of plating of 0.1 to 1.0; −, strong inhibition of phage growth, with an efficiency of plating of less than 10^{-4}.

Both *E. coli dnaK9 grpE*$^+$ and *dnaK90 grpE*$^+$ bacteria partially block wild-type λ growth. However, the inhibition of growth is not strong enough to be used as a diagnostic test in bacteriophage plating experiments. In contrast, both suppressors strongly inhibit the growth of λ grpE$^+$ transducing bacteriophage (Table 2). Since λ *dnaB*$^+$, λ *dnaJ*$^+$, and λ *dnaK*$^+$ transducing phages (similarly derived from λ L47.1 vector) grow better on these two strains than λ *grpE*$^+$ does, we suspect that overproduction of the *grpE*$^+$ gene product after infection interferes with bacteriophage λ growth. We have no clear explanation of why or how this phenomenon occurs.

Wild-type, *dnaK9 grpE*$^+$, and *dnaK90 grpE*$^+$ bacteria were transformed with pJK23 (22), a plasmid that overproduces the GrpE$^+$ protein, to further test the effect of the two *dnaK* suppressor mutations in relation to *E. coli* growth. We were unable to obtain any transformants at 30°C with either *E. coli dnaK9 grpE*$^+$ or *E. coli dnaK90 grpE*$^+$ bacteria as recipients, but we obtained >1,000 transformants with the wild-type parent strain. As a control, *E. coli dnaK9 grpE*$^+$ and *E. coli dnaK90 grpE*$^+$ bacteria were transformed at normal frequencies with pBR322. This result is consistent with the idea that *E. coli dnaK9 grpE*$^+$ and *E. coli dnaK90 grpE*$^+$ bacteria are sensitive to high levels of GrpE$^+$ protein and is consistent with the observed effect on λ growth.

λ *grpE*$^+$ plating properties (Table 2) provided us with a method of distinguishing our suppressor strains. *E. coli dnaK9 grpE280*, *dnaK9 grpE*$^+$, *dnaK90 grpE280*, and *dnaK90 grpE*$^+$ bacteria failed to plate λ *grpE*$^+$ transducing bacteriophage, while this bacteriophage plated with an efficiency of 0.5 to 1.0 on *dnaK*$^+$ *grpE*$^+$ and *dnaK*$^+$ *grpE280* bacteria (Table 2).

The *dnaK9* and *dnaK90* mutations resulted in a partial temperature sensitivity phenotype compared with the phenotype of the isogenic *dnaK*$^+$ parent. This sensitivity was seen at temperatures above 45°C on L-agar plates or at 44°C on T-agar plates prepared without NaCl.

***dnaK9* and *dnaK90* suppressors map in *dnaK* gene.** To confirm that the *dnaK9* and *dnaK90* suppressors do indeed

map in the *dnaK* gene, the following experiments were performed. *E. coli dnaK9 grpE*$^+$ and *dnaK90 grpE*$^+$ strains were transformed with two pBR322-derived plasmids. Both plasmids contain an *E. coli* DNA fragment carrying the *dnaK* gene. The first plasmid, pCG1, carries a 5.4-kilobase *Hin*dIII fragment containing the intact *dnaK*$^+$ gene (11). Plasmid pCG1 also carries a segment of the downstream *dnaJ* gene. This segment codes for the first 32 amino acids of the DnaJ protein (unpublished results). The second plasmid, pCG2, carries the same *E. coli* DNA fragment, except that the *dnaK* gene is inactivated by the insertion of an Ω element at its unique *Eco*RI site (4). The Ω element contains strong polar transcriptional and translational termination signals at each end and carries resistance to spectinomycin (27). This plasmid produces no full-length DnaK protein and does not complement *dnaK* mutant cells (data not shown).

The *E. coli dnaK9 grpE*$^+$ and *dnaK90 grpE*$^+$ strains transformed with pCG1 recovered their GrpE$^+$ λ plating phenotype; i.e., they plated λ *grpE*$^+$ transducing bacteriophage normally. However, when the cells were transformed with pCG2, they did not recover the GrpE$^+$ phenotype (Table 2). These results are strong evidence that both of these suppressor mutations map within the *dnaK* gene.

To monitor the effects of a single copy of either the *dnaK*$^+$ gene or the *dnaK*::Ω-disrupted gene on the expression of the *dnaK9* and *dnaK90* suppressor mutations, we repeated the plating assays after lysogenizing the *E. coli dnaK9 grpE*$^+$ and *dnaK90 grpE*$^+$ strains with a λ *imm*^{21} *dnaK*$^+$ transducing phage at the *att*$^\lambda$ site on the *E. coli* chromosome. The results of λ plating on these strains are shown in Table 2.

The simplest interpretation of these results is that (i) the *dnaK90* suppressor mutation maps in the *dnaK* gene and is recessive to the presence of a single copy of *dnaK*$^+$, and (ii) the *dnaK9* suppressor mutation is dominant to the presence of a single copy of *dnaK*$^+$. The results discussed above, which show that multiple copies of the *dnaK*$^+$ gene provided by pCG1 reversed the λ plating phenotype in *E. coli dnaK9* and *dnaK90*, while multiple copies provided by pCG2 did not, are evidence that *dnaK9* and *dnaK90* map in the *dnaK* gene. The possibility exists that the *dnaK9* mutation is not located in the *dnaK* gene and that suppression of its phenotype by the pCG1 plasmid is simply due to indirect effects of DnaK protein overproduction. This possibility is unlikely, however, because of the observed coimmunoprecipitation of the DnaK9 and GrpE280 mutant proteins (see below).

Bacteria with the genotype *dnaK90 grpE280*(λ *imm*^{21} *dnaK*$^+$) do not form colonies at 44°C, as might be expected if *dnaK*$^+$ is dominant over *dnaK90* with respect to both *E. coli* and λ growth. The existence of extragenic suppressors of the *grpE280* mutation which map in the *dnaK* gene provides strong evidence that the DnaK and GrpE proteins functionally interact in vivo.

Coimmunoprecipitation of DnaK and GrpE proteins from cell extracts. Immunoprecipitation studies of DnaK$^+$ protein from [^{35}S]methionine-labeled wild-type *E. coli* cell extracts with anti-DnaK$^+$ polyclonal antibodies showed significant precipitation of a 24,000-M_r protein (Fig. 2). The 24,000-M_r protein was subsequently identified by two-dimensional gel electrophoresis as the GrpE$^+$ protein, which is identical to HS protein B25.3 (2, 25). The GroEL protein present in the precipitations shown in Fig. 2 (its identity was verified by its position following two-dimensional gel electrophoresis) deserves comment. Since GroEL was seen in the preimmune serum precipitate, it was most likely precipitated by anti-GroEL antibodies already present in the serum. The GroEL protein has been shown to be the common antigen appearing

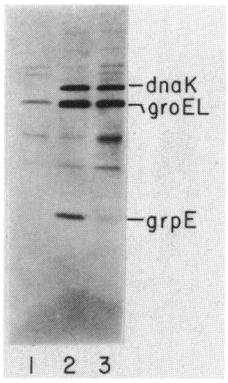

FIG. 2. Immunoprecipitation of ^{35}S-labeled cell extracts. Lane 1, Wild-type cell extract precipitated with control antibodies; lane 2, wild-type cell extract precipitated with anti-DnaK antibodies; lane 3, *grpE280* cell extract precipitated with anti-DnaK antibodies.

in bacterial infections of mammals (32). In addition, GroEL precipitates nonspecifically because of its inherent ability to aggregate. However, it is possible that the additional amount of GroEL protein found in anti-DnaK$^+$ antibody precipitations is coprecipitating through a specific interaction with DnaK$^+$ protein. We have not investigated this possibility further.

A cell extract prepared from [^{35}S]-labeled *E. coli grpE280* bacteria was used in the same anti-DnaK$^+$ polyclonal antibody assay. A drastic reduction in coprecipitation of GrpE280 protein was observed (Fig. 2, lane 3). Analysis of supernatants from the immunoprecipitation reactions showed that the majority of GrpE$^+$ protein coprecipitated with DnaK$^+$ protein, while most of the GrpE280 protein did not coprecipitate with DnaK$^+$ protein (data not shown). Hence, it appears that the *grpE280* mutation disrupts the coprecipitation observed between GrpE$^+$ and DnaK$^+$ proteins.

In analogous experiments with extracts from an *E. coli dnaK756* Tr$^-$ mutant, coprecipitation of GrpE$^+$ protein with the mutant DnaK756 protein was also greatly reduced (Fig. 3A, lane 3). *E. coli dnaK756* grows well at 30 and 37°C but fails to form colonies at 43.5°C. It produces a full-length DnaK protein with an isoelectric point which is more acidic than that of the DnaK$^+$ protein (12). One possible conclusion from these experiments is that the observed failure of GrpE280 and DnaK756 proteins to coprecipitate with DnaK$^+$ and GrpE$^+$, respectively, correlates with the Tr$^-$ bacterial phenotype.

To evaluate this possibility, we conducted immunoprecipitations of ^{35}S-labeled *E. coli dnaK9 grpE*$^+$ and *dnaK90 grpE*$^+$ cell extracts with anti-DnaK$^+$ antibodies. The amount of GrpE$^+$ protein coprecipitated with DnaK9 and DnaK90 proteins (Fig. 3B, lanes 1 and 3) was similar to that coprecipitated with the wild-type DnaK$^+$ protein (Fig. 3A, lane 1). Cell extracts were also prepared from the original *E. coli dnaK9 groE280* and *dnaK90 grpE280* strains. The amount of GrpE280 protein coprecipitating with DnaK9 (Fig. 3B, lane 2) was the same as the amount of GrpE$^+$ coprecipitating with DnaK$^+$ (Fig. 3A, lane 1). Coprecipitation of GrpE280 with DnaK90 protein was not found to be significant (Fig. 3B, lane 4).

Quantitation of the protein precipitated in the immunoprecipitation assays was done by densitometry of film exposures similar to those in Fig. 2 and 3. Since essentially all the DnaK$^+$ and GrpE$^+$ proteins were precipitated by the anti-

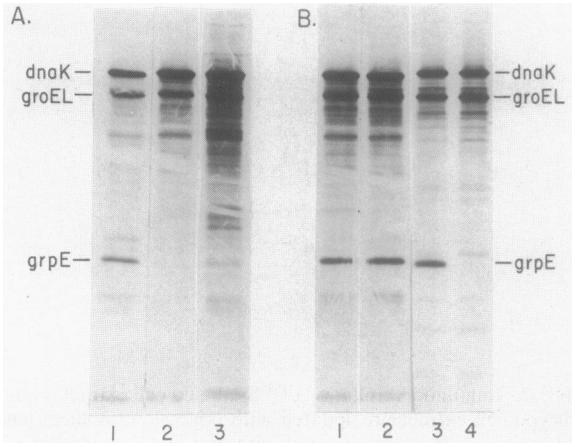

FIG. 3. Immunoprecipitation of wild-type and mutant cell extracts with anti-DnaK antibodies. (A) Lane 1, Wild-type extract; lane 2, *grpE280* extract; lane 3, *dnaK756* extract. (B) Lane 1, *dnaK9 grpE*$^+$ extract; lane 2, *dnaK9 grpE280* extract; lane 3, *dnaK90 grpE*$^+$ extract; lane 4, *dnaK90 grpE280* extract.

DnaK$^+$ antibodies (data not shown), we assume that the ratio of DnaK$^+$ to GrpE$^+$ reflects the in vivo ratio in *E. coli*. The relative density values obtained were adjusted for the cysteine and methionine contents of the DnaK$^+$ and GrpE$^+$ proteins predicted from the nucleotide sequences (4, 22). These values were used to calculate a ratio between the number of DnaK molecules and the number of GrpE molecules precipitated with the anti-DnaK antibodies. The average values calculated for the strains are as follows: *dnaK*$^+$ *grpE*$^+$, 2.1; *dnaK*$^+$ *grpE280*, 17.5; *dnaK9 grpE*$^+$, 1.9; and *dnaK9 grpE280*, 1.6. These values support the preliminary conclusions about the general pattern of association between DnaK and GrpE wild-type and mutant proteins. Approximately eight times more GrpE$^+$ than GrpE280 protein coprecipitated with DnaK$^+$ protein. This shows that DnaK$^+$ binds to GrpE$^+$ protein more efficiently than it binds to GrpE280 mutant protein. In turn, approximately 10 times less GrpE280 protein coprecipitated with DnaK$^+$ protein than with DnaK9 protein. This shows that the *dnaK9* mutation results in improved binding to GrpE280, to a level comparable to that between the DnaK$^+$ and GrpE$^+$ proteins.

An immunoprecipitation assay was performed with anti-GrpE$^+$ polyclonal antibodies. This experiment demonstrated that all the bacterial cultures used in this study synthesized comparable amounts of GrpE protein. In addition, when wild-type extracts were used, DnaK$^+$ protein coprecipitated with GrpE$^+$ protein in an approximate ratio of 1:2 (data not shown). This finding suggests either that the active form of GrpE$^+$ bound to DnaK$^+$ is a dimer or that only half of the GrpE$^+$ molecules which are precipitated are also bound to a DnaK$^+$ molecule. We favor the former interpretation because it is in agreement with the results of cross-linking experiments between the two purified proteins (M. Zylicz, D. Ang, and C. Georgopoulos, unpublished results).

DISCUSSION

The findings of these studies of the interaction between the *dnaK* and *grpE* gene products can be summarized as follows. (i) Mutations which suppress the Tr$^-$ phenotype of *E. coli grpE280* and which map in the *dnaK* gene have been isolated. This provides strong evidence that the DnaK$^+$ and GrpE$^+$ HS proteins functionally interact in vivo and substantiates previous results for purified proteins in vitro (36; C. Georgopoulos, K. Tilly, D. Ang, G. N. Chandrasekhar, O. Fayet, J. Spence, T. Ziegelhoffer, K. Liberek, and M. Zylicz, *in* M.-L. Pardue, J. Feramisco, and S. Lindquist, ed., *Stress-Induced Proteins*, in press). (ii) In a crude extract, GrpE$^+$ and DnaK$^+$ proteins coprecipitated with anti-DnaK$^+$ polyclonal antibodies, even in the presence of 1% Triton X-100 and 0.1% SDS, demonstrating a strong DnaK-GrpE interaction in vitro. (iii) The mutant DnaK756 and GrpE280 proteins showed greatly reduced coprecipitation with the GrpE$^+$ and DnaK$^+$ proteins, respectively. Thus, the Tr$^-$ phenotype of these mutant bacteria correlates with reduced DnaK and GrpE coprecipitation. (iv) DnaK9 suppressor protein activity was dominant over DnaK$^+$ activity, while DnaK90 protein activity was recessive. The *dnaK9* mutation appears to suppress the Tr$^-$ phenotype of the *grpE280* mutation by increasing the affinity of DnaK9 for GrpE280. This increased affinity may contribute to the observed dominance of *dnaK9* over *dnaK*$^+$. Although the *dnaK90* mutation did not reinstate the in vitro interaction with the mutant GrpE280 protein, it is possible that such an interaction may take place in vivo. We do not understand the mechanism by which *grpE90* compensates for the GrpE$^-$ phenotype. It could be that in vivo the DnaK90 and GrpE280 proteins do functionally interact and can carry out some of the functions necessary for *E. coli* survival at high temperature.

The *grpE280* mutation was originally selected for its ability to block λ DNA replication (28). Here we have shown that the *grpE280* mutation disrupts the interaction between the GrpE280 and DnaK$^+$ proteins. Since the *dnaK756* mutation also disrupts binding between the GrpE$^+$ and DnaK756 proteins and since both *grpE280* and *dnaK756* mutant bacteria fail to support λ growth, it seems likely that λ replication requires a functional interaction between these two proteins in vivo. Data from in vitro λ replication assays demonstrate that the presence of GrpE$^+$ protein allows DnaK$^+$ protein to function more efficiently in λ DNA replication (M. Zylicz, D. Ang, K. Liberek, and C. Georgopoulos, EMBO J., in press; Georgopoulos et al., in press).

Recent data from many laboratories on various members of the Hsp70 family of proteins have broadened our understanding of their functions. One of the proposed models for Hsp70 function involves an equilibrium reaction binding exposed, hydrophobic domains of other, potentially denatured proteins in solution, particularly during a response to stress. This binding by the Hsp70 protein may allow a change in conformation of the bound protein back to that of its native state. Subsequent release of the refolded protein into solution requires ATP hydrolysis (19, 26). Similarly, purified DnaK$^+$ protein has recently been shown to preferentially bind the unfolded form, rather than the native form, of another protein (C. Johnson, A. Cegielska, and C. Georgopoulos, manuscript in preparation). Damaged proteins with exposed denatured regions have been implicated as an HS response-inducing signal (1). The mechanism by which damaged proteins induce the HS response may involve a "saturation" of the *E. coli* protease system(s). Saturation of the protease system(s) could conceivably result in a lower rate of turnover of the *rpoH* gene product, σ^{32}, which may be subject to degradation by these proteases. It is known that σ^{32}, the sigma subunit of RNA polymerase which recognizes HS promoters, is highly unstable (3, 15, 29, 30a). The resulting stabilization of σ^{32} would lead to induction of transcription of HS genes. The mechanism by which DnaK$^+$

protein negatively regulates the *E. coli* HS response may involve a stabilization and refolding of heat-denatured proteins, thus eliminating the inducing signal.

The GrpE$^+$ protein may be modulating DnaK$^+$ activity in relation to ATP levels in the cell, since the DnaK$^+$-grpE$^+$ complex is disrupted by ATP in vitro (36). Lee et al. (18) noted a sharp decrease in ATP concentration in vivo 5 min after a shift from 23 to 42°C in *Salmonella typhimurium*. This time point is coincident with initiation of the negative modulation of HS protein synthesis seen in wild-type *E. coli* but not in *E. coli dnaK756* cells (30). Jones and Findley (17) showed that a similar decrease in ATP levels is required for acquisition of thermotolerance in *Tetrahymena* spp.; i.e., induction of HS proteins can occur without such a decrease in ATP, but no thermotolerance is observed. A similar study of HS protein induction in *E. coli* likewise documented induction without acquisition of thermotolerance (31). Since interaction of GrpE$^+$ protei with DnaK$^+$ is sensitive to ATP (36), the decrease in ATP may shift the equilibrium toward greater frequency of GrpE$^+$ interaction with DnaK$^+$, and this association may somehow modify DnaK$^+$ activity.

Possible evidence of a eucaryotic protein acting in conjunction with Hsp70, the eucaryotic analog of DnaK, has been provided by studies of yeast cells. Chirico et al. (7) and Deshaies (10) recently showed that two yeast DnaK-like proteins stimulated the translocation of precursor polypeptides into microsomes in the presence of ATP. However, translocation of unfolded polypeptides occurred in the absence of the DnaK-like proteins, suggesting that their role is to unfold the precursor polypeptides. While this translocation activity was being purified, another factor(s) was also found to be involved. In the absence of this additional activity, less efficient translocation was observed. This factor may have GrpE$^+$-like activity.

A view of how GrpE$^+$ protein might modify DnaK$^+$ activity during HS is suggested by results for λ DNA replication. In this system, the GrpE$^+$ protein is observed to increase the efficiency of DnaK$^+$ activity in dissociating DnaB from λ P protein (Georgopoulos et al., in press; Zylicz et al.,in press). During protracted heat stress, DnaK$^+$ action conceivably would be less efficient if it released the proteins it "repaired" as soon as they were refolded; i.e., if refolded heal-labile proteins were released while the temperature was still high, they would become denatured again. Perhaps the binding of GrpE$^+$ protein modifies DnaK$^+$ activity by inhibiting release of the repaired protein. The equilibrium shift toward a GrpE$^+$-DnaK$^+$ complex at low levels of ATP may increase the number of DnaK$^+$ molecules which maintain stabilization of heat-sensitive proteins. When ATP in the cell resumes normal levels, the equilibrium would then shift toward dissociation of GrpE$^+$ from DnaK$^+$, resulting in release of the repaired, protected proteins.

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