Interaction between the Nucleotide Exchange Factor Mge1 and the Mitochondrial Hsp70 Ssc1*

Sayuri Sakuragi‡, Qinglian Liu, and Elizabeth Craig§
From the Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Function of Hsp70s such as DnaK of the Escherichia coli cytoplasm and Ssc1 of the mitochondrial matrix of Saccharomyces cerevisiae requires the nucleotide release factors, GrpE and Mge1, respectively. A loop, which protrudes from domain IA of the DnaK ATPase domain, is one of six sites of interaction revealed in the GrpE:DnaK co-crystal structure and has been implicated as a functionally important site in both DnaK and Ssc1. Alanine substitutions for the amino acids (Lys-108 and Arg-213 of Mge1) predicted to interact with the Hsp70 loop were analyzed. Mge1 having both substitutions was able to stimulate nucleotide release from Ssc1 and function in refolding of denatured luciferase, albeit higher concentrations of mutant protein than wild-type protein were required. In vitro and in vivo assays using K108A/R213A Mge1 and Ssc1 indicated that the disruption of contact at this site destabilized the interaction between the two proteins. We propose that the direct interaction between the loop of Ssc1 and Mge1 is not required to effect nucleotide release but plays a role in stabilization of the Mge1-Ssc1 interaction. The robust growth of the K108A/R213A MGE1 mutant suggests that the interaction between Mge1 and Ssc1 is tighter than required for function in vivo.

Molecular chaperones such as members of the 70-kDa class (Hsp70s)1 bind to nonnative conformations of proteins thus facilitating cellular processes such as folding of proteins and their translocation across membranes (1, 2). Although the C-terminal 28-kDa region of Hsp70s binds unfolded polypeptides, the highly conserved N-terminal 44-kDa domain regulates that binding through its interaction with adenine nucleotides. It is thought that Hsp70 proteins, like many GTPases, have a two-state conformation. When an ADP molecule is bound to the nucleotide-binding site, the Hsp70 exhibits stable peptide binding; when ATP is bound, binding of peptide is relatively unstable (3, 4). The 44-kDa domain also has a low intrinsic ATPase activity (5); therefore, ATP hydrolysis converts Hsp70 to the form having a relatively stable interaction with unfolded proteins. However, exchange of ADP for ATP results in transient interactions.

Nucleotide release factors are essential components of at least some Hsp70 chaperone machines. GrpE of Escherichia coli was the first nucleotide release factor identified (6). Release of ADP from a DnaK-ADP complex can be increased up to 5000-fold by GrpE action, resulting in a reduction in the affinity of DnaK for ADP of about 200-fold (7). A related protein, Mge1, an essential protein of yeast mitochondria (8–10), has recently been shown to stimulate nucleotide release from the mitochondrial Hsp70, Ssc1 (11, 12). Interaction of both proteins with their respective Hsp70 is resistant to high salt but disrupted by addition of ATP (11, 13).

GrpE binds stably to the 44-kDa N-terminal domain, although there are likely interactions with the C-terminal region of the protein as well (14, 15). GrpE is a homodimer that binds a single DnaK molecule. The binding is asymmetric, with one of the monomers providing the vast majority of interactive sites with the 44-kDa N terminus. The 44-kDa is composed of two large domains, each of which is composed of two subdomains. Subdomains IA and IIA lie at the base of the deep ATP-binding cleft; subdomains IB and IIB form the ATP-ADP-binding sites. There are six areas of interaction spread across one face of the 44-kDa fragment. Comparison of GrpE-related sequences suggests that the structures of GrpE and Mge1 are similar, as are their interactions with Hsp70s (16). In fact, Mge1 is able to substitute for GrpE in E. coli (17).

One of these six sites of interaction between GrpE and DnaK is a conserved loop (amino acids 28–34 and 56–62 in domain IA of DnaK and Ssc1, respectively) which was found to be important in GrpE:DnaK and Mge1:Ssc1 interactions (11, 14, 15). Designated as site IV in the published structure, this loop is not a major site in terms of overall contact area in the GrpE:DnaK co-crystal structure (15) (Fig. 1). However, a mutation that resulted in an alanine instead of a glycine at position 32 (G32A) caused a destabilization of the interaction between DnaK and GrpE. The G32A mutant protein was unable to rescue either the growth defect of cells lacking wild-type DnaK or their inability to replicate phage λ (14). It was proposed that this destabilization of the physical interaction prevented GrpE from facilitating nucleotide release, hence the null phenotype. The analysis of the analogous alteration in Ssc1 (G60D) presented a more confusing picture. The mutant Ssc1 stably bound Mge1, but nucleotide release was not stimulated by this interaction (11). The ineffectual binding of Mge1 to the G60D mutant suggested that interaction of Mge1 with this loop of region IV was important for triggering nucleotide release.

To address the role of the loop region of Ssc1 in the mechanism of nucleotide release we constructed and analyzed alanine substitution mutants expected to alleviate all Mge1 interaction with Ssc1 at site IV. The results of the analysis indicate that this site of interaction between Mge1 and Ssc1 plays a role in

* This work was supported in part by National Institutes of Health Grant GM27870 (to E. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by fellowships from the Japan Society for Promotion of Science and the Yoshida Foundation. Present address: The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
§ To whom correspondence should be addressed. Tel.: 608-263-7105; Fax: 608-262-5253; E-mail: e craig@facstaff.wisc.edu.
1 The abbreviations used are: Hsp70, 70-kDa class heat shock protein; PCR, polymerase chain reaction; MOPS, 4-morpholino-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

This paper is available on line at http://www.jbc.org

Received for publication, December 31, 1998

Printed in U.S.A.

Vol. 274, No. 16, Issue of April 16, pp. 11275–11282, 1999

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
PROTEIN EXPRESSION AND PURIFICATION

Strains and Plasmids

The *Saccharomyces cerevisiae* and *E. coli* strains used in this study are listed in Table I. LB medium was prepared as described (18) and supplemented with 100 μg/ml ampicillin, 100 μg/ml kanamycin, and/or 25 μg/ml chloramphenicol where appropriate. Yeast YPD and minimal media were prepared as described (19).

**E. coli Plasmids**—pBW401 is a low copy plasmid, pWSK29 (20), containing the wild-type *grpE* gene (21). A control plasmid, pWSK29ΔΔE, was constructed by cleaving pBW401 with SauI and KcoRI and religating to remove the *grpE* gene. Mutant *grpE* genes were constructed and cloned into pWSK29 replacing the wild-type sequences with the mutant sequences, generating pWSK29grpEΔE. pWSK29grpEΔE was transfected into *E. coli* OD212, DA259, and DA810 which were grown at 30 °C and induced for expression of the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and disrupted with a French pressure cell, and the soluble extract was incubated with glutathione-agarose beads (Sigma). After washing, the beads were incubated with thrombin (Sigma T3010); the cleavage product was collected. The protein preparations were judged to be greater than 95% pure by Coomassie Blue staining. The protein concentrations of preparations were determined using the Bradford assay (Bio-Rad) using ovalbumin as a standard.

**S. cerevisiae Plasmids**—The *S. cerevisiae* plasmids used in the purification of Mge1, pGEX-KT-MGE1, and the GST-SCS1 fusion, pRD656CSS-SCS1, were described previously (11). Mutant MGE1 genes were cloned into pGEX-KT-MGE1 to generate the plasmids pGEX-KT-mge1-K108A, pGEX-KT-mge1-R213A, and pGEX-KT-mge1-K108A/R213A which were used to purify mutant Mge1 proteins. Plasmid pRS314-SCS1-MGE1 was generated from pRS314-SCS1 (11).

Site-directed Mutagenesis

Mutations K108A, R213A, and K108A/R213A in *S. cerevisiae* were prepared with appropriate antibodies. Growth of transformants was tested by plating serial dilutions of cultures onto plates containing L broth supplemented with the appropriate antibodies; growth was observed after overnight incubation at various temperatures.

DnaJ expression was induced in LB medium carrying the plasmid pGEX263 as described by Karzai and McCormick (22). DnaJ purified from *E. coli* strain OD3479 carrying the expression plasmid pGEX263 was expressed in *S. cerevisiae* as described by Baas et al. (24) using a DEAE-Sepharose column, followed by ATP-agarose and hydroxyapatite chromatography.

Luciferase Refolding Assay

1 μl of a 1 mg/ml solution of luciferase (Sigma) dissolved in 1 mM glycylglycine was added to 5.4 μl of unfolding buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, and 6 mM guanidine HCl) and incubated 1 h at room temperature. 0.078 μg of the unfolded luciferase (1 μl) was mixed with 62 μl of refolding buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 1 mM ATP) which was 0.8 μM DnaK and varying amounts of DnaJ or Mge1 and incubated at room temperature. At various times 2 μl of the fractions were rapidly diluted 1:25 with dilution buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin), injected into a luminometer (Berthold), and the activity of luciferase was measured.

Co-immunoprecipitation Assays—Mitochondria were prepared from BM37-7 containing various pRS314-SCS1-MGE1 plasmids as described previously (26) and stored at −70 °C until use. 100 μg of mitochondria was suspended in P80 buffer (10 mM MOPS-KOH (pH 7.2), 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 3% (w/v) bovine serum albumin) and then centrifuged at 14,000 rpm for 7 min at 4 °C. Pellets were lysed on ice by incubation in lysis buffer A (250 mM sucrose, 80 mM KCl, 20 mM MOPS-KOH (pH 7.2), 0.2% Triton X-100, and 5 mM EDTA) or lysis buffer B (250 mM sucrose, 80 mM KCl, 20 mM MOPS-KOH (pH 7.2), 0.2% Triton X-100, 3 mM Mg(OAc)₂, and 1 mM ATP) for 15 min. Lysates were centrifuged for 10 min at 14,000 rpm at 4 °C. Aliquots were mixed with 10 μl (bed volume) of protein A-Sepharose beads (Sigma) cross-linked with purified anti-Scs1 antibody and incubated with the supernatants of the lysates for 1 h at 4 °C. The beads were washed with lysis buffer A or B 3 times. Beads were resuspended in 2× Laemmli’s buffer (24 mM Tris-HCl (pH 6.8), 10% glycerol, 0.8% SDS, 5.76 mM β-mercaptoethanol, and 0.04% bromphenol blue). The resuspended proteins were separated by SDS-12.5% PAGE, blotted, and probed with antibodies against Mge1 and Scs1. ECL Western blots (Amersham Pharmacia Biotech) were performed according to manufacturer’s suggestions.

Interaction of Nucleotide Exchange Factor Mge1 and Hsp70 Ssc1

**Table I**

| Strain | Genotype and phenotype | Source or Ref. |
|--------|------------------------|---------------|
| E. coli | PK101                  |               |
|        | OD212                  | AM267, dnak323 grpE::oegma-camR |
|        | DA259                  | C600, thr::Tn10 grpEX::oegma-camR kanR |
|        | DA810                  | B178, pheA::Tn10 grpE280 |
|        | RLM569                 | C600, hisdR leu-pro-lac-tonA rpsL (strR) recA |
| S. cerevisiae | BJ3497               | pep4::HIS3 ura3–5 his3Δ200 |
|        | JD100                  | lys2 ura3–5 Δtrp1 leu2–3, 112 his3–11,15 ade2–1 can1–100 |
|        | BM37–7                 | GAL12 met2–Δ1 lys2–Δ2 SCS1Δclass::LEU2 |
|        | mge1::T (pRS316R-SCS1-MGE1) | This study |
an equal volume of 0.1 M wild-type or mutant Ssc1 was incubated with 100 antibodies as described above. Buffer E at 4 °C. 70-
min. The reaction was chilled on ice and immediately loaded onto a NICK column (Amersham Pharmacia Biotech) pre-equilibrated with 10 mM Mg(OAc)2 and 10 mM ATP. Equivalent samples were collected at various stages, separated by SDS-PAGE, and probed with antibodies as described above.

**In Vitro Mge1 Binding Assay**

GST-Ssc1 fusion protein was immobilized on glutathione-agarose beads as described previously (11). After extensive washing with buffer D (25 mM Hepes-KOH (pH 7.5), 100 mM KCl, and 11 mM Mg(OAc)2 containing 25 μM ATP at 30 °C for 15 min. The reaction was chilled on ice and immediately loaded onto a NICK column (Amersham Pharmacia Biotech) pre-equilibrated with buffer E at 4 °C. 70-μl fractions were collected. The first peak of radioactivity that corresponded to the Ssc1-ATP complex was pooled, adjusted to 10% glycerol, aliquoted, and stored at -70 °C. For a single turnover assay, a 10-μl aliquot of the Ssc1-ATP complex was thawed and immediately mixed with an equal volume of buffer E containing other components such as Mge1, as indicated, and incubated at 30 °C. At the indicated times the reaction was stopped and the percent conversion to ADP determined (11).

**Results**

**Alteration of Residues of Mge1 (Lys-108 and Arg-213) Predicted to Interact with Ssc1 Has Little Phenotypic Effect in Vivo—**The loop encoded by amino acids 28 to 34. Individual interactions between GrpE (on the left) and DnaK (on the right) are indicated by the dashed lines. Heavy dashed lines indicate two interactions, one with the oxygen and one with the carbon of the carbonyl group of Glu-31. Designations in parentheses indicate the analogous residues in Ssc1 and Mge1. Information for figure taken from Harrison et al. (15).

**Single Turnover ATPase Assay**

Complex formation and single turnover assays were performed essentially as described previously (11) with several modifications. 25 μg of wild-type or mutant Ssc1 was incubated with 100 μCi of [α-32P]ATP (DuPont, 3000 Ci/mmol) in buffer E containing 25 μM ATP at 30 °C for 15 min. The reaction was chilled on ice and immediately loaded onto a NICK column (Amersham Pharmacia Biotech) pre-equilibrated with buffer E at 4 °C. 70-μl fractions were collected. The first peak of radioactivity that corresponded to the Ssc1-ATP complex was pooled, adjusted to 10% glycerol, aliquoted, and stored at -70 °C. For a single turnover assay, a 10-μl aliquot of the Ssc1-ATP complex was thawed and immediately mixed with an equal volume of buffer E containing other components such as Mge1, as indicated, and incubated at 30 °C. At the indicated times the reaction was stopped and the percent conversion to ADP determined (11).

Since no mutations in MGE1 were identified that could overcome the defect of the SSC1 G60D mutation, we decided to test directly the importance of residue Arg-213. Six single amino acid substitutions were tested by transforming a plasmid carrying the mutant MGE1 genes into a strain having a deletion of MGE1 on the chromosome and a wild-type copy of MGE1 on aURA3-containing plasmid. The ability of the mutant MGE1 to rescue growth of a mge1 deletion mutant was monitored by streaking on 5-fluoroorotic acid-containing media which inhibits growth of cells expressing the URA3 gene product. Therefore only cells carrying mutant genes which were able to support growth in the absence of expression of wild-type MGE1 were able to form colonies in this test. Of the six mutations tested, five (R213A, R213L, R213C, R213D, and R213H) allowed wild-type growth at 30 °C (Fig. 2 and data not shown). Only R213P was nonfunctional. The effect of the proline substitution is not surprising because of the propensity of proline to substantially disrupt structure.

**Inspection of the GrpE:DnaK structure suggests that the only other interaction between this loop and Mge1 occurs between Lys-108 of Mge1 and Glu-56 of Ssc1. These residues are identical in GrpE and DnaK. Therefore we constructed mutations at the Lys-108 codon of MGE1 by site-directed mutagenesis and tested their ability to function. Alanine was chosen as a substitution because it would be expected to disrupt the normal interaction with the side chain of Glu-56 in Ssc1; glutamic acid was selected since it would change a positive to a negative charge at this position, and would be predicted to juxtapose two amino acids having the same charge. However, strains carrying either mutant gene grew as well as wild-type cells at a variety of temperatures, utilizing both fermentable and nonfermentable carbon sources (Fig. 2 and data not shown).**

Since each alanine substitution alone caused no detectable phenotype, we constructed the double mutant K108A/R213A MGE1 to test the effect of alanines at both positions. Based on the GrpE:DnaK structure these mutations would be expected to obviate all interactions between Mge1 and the loop of Ssc1 since the side chains of the Mge1 amino acids are the interactive sites (Fig. 1). This double mutant was viable, growing as well as wild-type at 30 °C and only slightly compromised for growth at 37 °C on both glucose- and glycerol-based medium (Fig. 2 and data not shown). These results suggest that the physical interaction between Mge1 and the loop of Ssc1 is not essential for Mge1 to function as a nucleotide exchange factor.

**Alanine Substitution of Residues Lys-82 and Arg-183 in GrpE That Interact with DnaK Do Not Affect in Vivo Function—**The interpretation of the results presented above assumes that the Mge1-Ssc1 interaction is the same as the GrpE-DnaK interaction. Although the amino acid sequences of the two pairs of proteins are related and the amino acids relevant to the interaction discussed here are identical, it is not known whether the two structures are completely analogous. There-
fore, we decided to change amino acids at the positions in GrpE (Lys-82 and Arg-183), which are analogous to those tested in Mge1, to alanines and test the effect of these substitutions on GrpE function. Three systems have been established for analysis of mutations in grpE, an essential gene as follows: the rescue of the temperature-sensitive growth of 1) the grpE mutation, grpE280 (17, 27); 2) a strain carrying both a deletion of the grpE gene and the dnaK332 compensatory allele which allows growth at lower temperatures in the absence of GrpE (17); and 3) a strain carrying an extragenic suppressor of the grpE deletion mutation (28). We tested the single and the double mutants by transforming low copy plasmids carrying each mutant gene into appropriate strains and testing for growth at 30, 37, 42, and 43 °C. For comparison, each test strain was also transformed with a plasmid containing wild-type protein, requiring about a 10-fold excess to have the same effect as wild-type protein.

Effect of Alterations of Mge1 Residues Lys-108 and Arg-213 on Nucleotide Release Activity—In the case of the double mutant K108A/R213A, we were able to stimulate the release of ATP from Ssc1 as the nucleotide release activity is thought to be the essential function of GrpE. Mge1, like the analogous residues in Mge1, are not essential for in vivo function. Hence, since the nucleotide release activity is thought to be the essential function of GrpE, these results suggest that these residues are not essential for the nucleotide release activity of these factors. In addition, these results also support the idea that the Mge1-Ssc1 interaction and the GrpE-DnaK interaction are the same.

Effect of Alterations of Mge1 Residues Lys-108 and Arg-213 on Nucleotide Release Activity—Since the double mutant K108A/R213A allowed nearly wild-type growth, we tested its ability to stimulate the release of ATP from Ssc1 as the nucleotide release activity is thought to be the essential function of GrpE/Mge1. To assess release activity we used a single turnover ATPase assay, monitoring the hydrolysis of radiolabeled ATP prebound to Ssc1 (11). This assay is based on the idea that release of nucleotide from Ssc1 facilitated by Mge1 will cause a decrease in hydrolysis of the radiolabeled ATP. Since an excess of unlabeled ATP is included in the reaction, released radiolabeled nucleotide will only rarely be rebound to Ssc1. The radiolabeled adenine nucleotide content of the isolated nucleotide-Ssc1 complex was about 80% ATP and 20% ADP (Fig. 4A). About 45% of the ATP bound to Ssc1 was hydrolyzed within 10 min at 30 °C. As expected, addition of nonradioactive ATP to the reaction had little effect on the hydrolysis of the prebound ATP indicating that the nucleotide remains bound throughout the time course of the reaction. However, as previously reported (11), addition of wild-type Mge1 reduced the hydrolysis of radiolabeled ATP when unlabeled nucleotide was added to prevent rebinding of released radiolabeled nucleotide (Fig. 4B). For example, the presence of Mge1 at a concentration of 8 μM reduced ATP hydrolysis by about 70% at 10 min incubation. A significant lowering of hydrolysis was observed upon addition of Mge1 to concentrations as low as 2 μM; at this concentration of Mge1 a 28% reduction was observed.
one immobilized on beads (11). As expected, purified wild-type Mge1 quantitatively bound to immobilized Ssc1, remained stably bound even in the presence of 1 M KCl, and was released in the presence of ATP (Fig. 6). K108A behaved as wild-type in this assay. R213A, however, did not bind as well as wild type; about 60% was retained on the column with the remaining 40% detected in the flow-through fraction. No binding of K108A/R213A was observed in this assay; all the mutant Mge1 protein was in the flow-through fraction.

This lack of detectable interaction of R213A was surprising...
since this mutant protein was nearly as effective as wild-type protein in supporting cellular growth even at high temperatures. Therefore, we wanted to assess the ability of these mutant Mge1 proteins to interact with Ssc1. ATPase domain of Hsp70 in the nucleotide release activity of Mge1. Three types of experiments were used to assess the effect of alterations of amino acids of Mge1 that interact with Ssc1. Even though this technique demands that an interaction be stable enough to allow retention of the complex

**FIG. 6.** Effect of mge1 amino acid substitutions on binding to Ssc1. A, GST-Ssc1 was immobilized on glutathione-agarose beads. Purified wild-type (Mge1) or mutant proteins (K108A, R213A, and K108A/R213A) were mixed with the beads which were then extensively washed and eluted with 1 mM ATP. L, total amount of Mge1 loaded onto beads; F, flow-through fraction; S, salt wash with 1 M NaCl in buffer; E, eluted with 10 mM ATP; B, beads after elution with ATP. B, 2 μg of wild-type and mutant Mge1 preparations were subjected to SDS-PAGE, and the gels were stained with Coomassie Blue.

**FIG. 7.** Co-immunoprecipitation of Mge1 with Ssc1 in mitochondria. A, isolated mitochondria were disrupted and subjected to immunoprecipitation with Ssc1-specific antibody in the presence of either ATP and Mg²⁺ (+ATP) or EDTA (+EDTA). The precipitates were resuspended and aliquots separated by SDS-PAGE. The gels were subjected to immuno blot analysis using Mge1-specific antibody to compare the amount of Mge1 protein in each sample. Lane 1, wild-type (WT) Mge1; lane 2, K108A; lane 3, R213A; lane 4 (K108A/R213A). R213A/K108A mitochondria, respectively, compared with wild type. These results from analysis of mitochondria are in agreement with the in vitro data and suggest that the single mutant R213A protein has an altered interaction with Ssc1, and the double mutant K108A/R213A protein has a more pronounced defect. However, while no interaction was detected with K108A/R213A in the in vitro assay, an interaction, although reduced, was detected in the co-immunoprecipitation experiments.

**DISCUSSION**

How nucleotide release factors of the GrpE/Mge1 class act mechanistically to facilitate release of nucleotide from Hsp70s is not resolved. Experiments reported here were designed to elucidate the role of a conserved exposed loop in domain IA of the ATPase domain of Hsp70 in the nucleotide release activity of Mge1. Three types of experiments were used to assess the effect of alterations of amino acids of Mge1 that interact with this loop (Lys-108 and Arg-213) as follows: in vitro biochemical assays to assess nucleotide release activity; in vivo and in vitro assays to appraise the physical interaction between the two proteins; and cell growth determinations to test the effect of these changes in vivo. Results of single turnover ATP hydrolysis and luciferase refolding assays indicated a functional inactivation of K108A/R213A with Ssc1. But, 8–10-fold higher concentrations of mutant compared with wild-type protein were required for similar effects.

Experiments we performed with purified proteins failed to detect a physical interaction between Lys-108/Arg-213 Mge1 and Ssc1. Even though this technique demands that an interaction be stable enough to allow retention of the complex
through an extensive series of washings, such a dramatic effect was surprising given what is known about the GrpE-Dnak interaction, especially since the alterations of these two exposed charged residues to alanines would not be expected to change the structure of the nucleotide release factor. The GrpE:Dnak co-crystal structure solved by Harrison et al. (15) revealed 159 interactions between the two proteins covering 2800 Å². Only 14 involved Lys-82 and Arg-183 (Lys-108 and Arg-213 in Mge1, respectively). Hence site IV which encompasses these interactions was considered a minor interaction site. Of the 14 interactions entered into by these two charged residues in GrpE with Dnak, 4 involve Lys-82 (Lys-108 in Mge1) and 10 involve Arg-183 (Arg-213 in Mge1). Consistent with this information K108A Mge1 had interactions with Ssc1 that were indistinguishable from that of wild-type protein. R213A Mge1 showed decreased but detectable interaction. Therefore, only the double mutant protein had a severe defect. Alternative biophysical assays will need to be developed in order to determine how greatly the affinity of Mge1 and Ssc1 is affected by these amino acid substitutions.

The results of the co-immunoprecipitation assays carried out with isolated mitochondria showed trends similar to those found with the purified proteins. However, the magnitude of the differences in interaction of K108A/R213A with Ssc1 was less in the immunoprecipitation assay. Although only about 14% as much K108A/R213A Mge1 as wild-type Mge1 was co-immunoprecipitated after preincubation at 37 °C, when the mitochondria were maintained at 4 °C about 50% as much mutant Mge1 associated with Ssc1 as wild-type Mge1. The reason for this difference in the results between the two types of assays is not clear. The conditions may be more stringent in the in vitro assay compared with the in organelar assay. Alternatively, it is possible that other proteins play some role in stabilizing the Mge1-Ssc1 interaction, as Ssc1 is known to interact with a number of other proteins in the mitochondrial matrix including Tim44 (29, 30), Tim17 (31), and Mdj1 (32). However, together these results suggest that the mutant Mge1 does physically interact with Ssc1 and facilitate nucleotide release, but the physical interaction is significantly less stable than the wild-type interaction, and thus higher concentrations of mutant protein are required for the same degree of functional efficacy.

The fact that the K108A/R213A mutation is able to facilitate nucleotide release argues that physical interaction between Mge1 and the loop of Ssc1 formed between amino acids 56 and 62 is not required for Mge1 to mechanistically affect nucleotide release. These results are consistent with the idea proposed by Harrison et al. (15) that binding of GrpE (and Mge1) exerts its effect on nucleotide release by shifting domain IIB of Hsp70 relative to the rest of the ATPase domain. Comparison of the crystal structure of the ATPase domain of Hsc70 with the GrpE:Dnak co-crystal structure suggests a mechanism of stimulation of nucleotide release from Hsc70. Although most aspects of the structure coincide very closely, domain IIB in the GrpE:Dnak structure is displaced about 14° relative to the Hsc70 structure. Three Dnak residues that interact with the adenine and ribose rings of ADP are displaced 2–3 Å. Such a displacement would be expected to significantly weaken the interaction with nucleotide. According to this scenario, the nucleotide-binding site is disrupted by the mechanical opening of the Dnak structure. Our results indicate that although the physical interaction with the loop formed by amino acids 56–62 is not required for such an opening of the ATPase domain of Ssc1, a certain stability of Mge1 binding is required to obtain the level of stimulatory effect achieved by wild-type Mge1. Hence increasing the concentration of K108A/R213A is able to attain wild-type levels of activity in the in vitro assays because it overcomes the weaker interaction caused by the mutations.

Although the data presented here indicate that physical interaction with the loop of Ssc1 is not needed for Mge1 to facilitate nucleotide release, certain amino acid substitutions in this loop result in a null phenotype. For example, G60D Ssc1 cannot rescue growth of ssc1 cells. However, G60D Ssc1 binds Mge1 stably but does not stimulate nucleotide release as measured by the single turnover ATPase assay (11). If interaction between Mge1 and the Ssc1 loop is not required for nucleotide release, what is the explanation for the null phenotype? The null mutation may affect the structure of Ssc1 in a manner that disrupts its ability to function without altering the stability of binding of Mge1. However, up to this point the only defect of G60D Ssc1 we observed is lack of stimulation of nucleotide release upon binding of Mge1. The steady state and single turnover ATPase activity of mutant and wild-type protein are similar, as are the Kₘ for interaction with ATP (data not shown). Perhaps the normal loop structure is important for maintaining the ATPase domain of Hsp70 in a conformation competent for Mge1 action but not other biochemical properties. Further experiments will be required to understand the requirements for the functional interaction between Hsp70 and its nucleotide release factor, a critical interaction for many Hsp70s.

Although Mge1 is an essential nucleotide release factor for Ssc1, the results presented here suggest that the release activity normally present in vivo is more than necessary to allow wild-type growth rates under a variety of laboratory conditions. This conclusion is based on the observation that alteration of 2 residues of Mge1 predicted to interact with Ssc1, Lys-108 and Arg-213, causes a significantly reduced stability of the interaction between the two proteins, both in an in vitro assay using purified components and in co-immunoprecipitation experiments using isolated mitochondria. However, only at temperatures at the high end of the growth range, such as 37 °C, was growth compromised, and this difference was only slight. Ssc1, like other molecular chaperones, is involved in assisting the renaturation of proteins that are partially unfolded at higher temperatures. The effect on growth at 37 °C, therefore, may be due to a higher demand for Ssc1:Mge1 function as temperature increases. Alternatively, the slight temperature sensitivity may be due to further destabilization of the Mge1-Ssc1 interaction at the higher temperatures as indicated by the co-immunoprecipitation experiments.

Acknowledgments—We thank Roger McMacken, Carol Gross, Costa Georgopoulos, and Debbie Ang for generously providing strains and plasmids and Cindy Voisine and Julie Davis for thoughtful discussions during the course of this work. We also thank Bingjie Miao for searches for suppressors and helpful discussions during the early stages of this work and William Walter for help with many of the experiments.

REFERENCES
1. Hartl, F. U. (1996) Nature 381, 571–580
2. Johnson, J. L., and Craig, E. A. (1997) Cell 90, 201–204
3. Schmid, D., Baici, A., Gehring, H., and Christen, P. (1994) Science 263, 971–973
4. Thaysen, H., Schuster, H. P., Packsies, L., Bukau, B., and Reinstein, J. (1996) J. Mol. Biol. 263, 657–670
5. McKay, D., Wilbanks, S., Flaherty, K., Ha, J.-H., O’Brien, M., and Shirvanee, L. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperones (Mormito, R., Tissieres, A., and Georgopoulos, C., eds) pp. 153–178, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6. Liberek, K., Marszalek, J., Ang, D., and Georgopoulos, C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2574–2578
7. Packsies, L., Thaysen, H., Buchberger, A., Bukau, B., Goody, R., and Reinstein, J. (1997) Biochemistry 36, 3417–3422
8. Lalaraya, S., Gambill, B. D., and Craig, E. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6481–6485
9. Bölger, L., Deloche, O., Glick, B., Georgopoulos, C., Jeno, P., Kronidou, N., Horst, M., Morishima, N., and Schatz, G. (1994) EMBO J. 13, 1998–2006
10. Ikeda, E., Yoshiida, S., Mitsuzawa, H., Uno, I., and Tóh-e, A. (1994) FEBS Lett. 339, 265–268
Interaction of Nucleotide Exchange Factor Mge1 and Hsp70 Ssc1

11. Miao, B., Davis, J. E., and Craig, E. A. (1997) *J. Mol. Biol.* **265**, 541–552
12. Dekker, P. J., and Pfanner, N. (1997) *J. Mol. Biol.* **270**, 321–327
13. Zylicz, M., Ang, D., and Georgopoulos, C. (1987) *J. Biol. Chem.* **262**, 17437–17442
14. Buchberger, A., Schroder, H., Buttner, M., Valencia, A., and Bukau, B. (1994) *Nat. Struct. Biol.* **1**, 95–101
15. Harrison, C. J., Hayter-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. (1997) *Science* **276**, 431–435
16. DeLoche, O., and Georgopoulos, C. (1996) *J. Biol. Chem.* **271**, 23960–23966
17. DeLoche, O., Kelley, W., and Georgopoulos, C. (1997) *J. Bacteriol.* **179**, 6066–6075
18. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J. G., Smith, J., and Struhl, K. (1997) *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 8.5.7–8.5.9, John Wiley & Sons, Inc., New York
24. Zylicz, M., Yamamoto, T., McKittrick, S., and Georgopolous, C. (1985) *J. Biol. Chem.* **260**, 7591–7598
25. Kamath-Loeb, A., Lu, C. Z., Suh, W.-C., Lonetto, M., and Gross, C. (1995) *J. Bacteriol.* **270**, 30551–30559
26. Gambill, B. D., Voos, W., Kang, P. J., Miao, B., Langer, T., Craig, E. A., and Pfanner, N. (1993) *J. Cell Biol.* **123**, 109–117
27. Ang, D., Chandrasekhar, G. N., Zylicz, M., and Georgopolous, C. (1986) *J. Bacteriol.* **167**, 25–29
28. Ang, D., and Georgopolous, C. (1989) *J. Bacteriol.* **171**, 2748–2755
29. Voos, W., Ahsen, O., Muller, H., Guiard, B., Rassow, J., and Pfanner, N. (1996) *EMBO J.* **15**, 2668–2677
30. Horst, M., Oppliger, W., Feifel, B., Schatz, G., and Glick, B. (1996) *Protein Sci.* **5**, 759–767
31. Bomer, U., Meijer, M., Maarse, A. C., Honlinger, A., Dekker, P. J. T., Pfanner, N., and Rassow, J. (1997) *EMBO J.* **16**, 2205–2216
32. Horst, M., Oppliger, W., Rospert, S., Schonfeld, H.-J., Schatz, G., and Azem, A. (1997) *EMBO J.* **16**, 1842–1849
33. Kang, P. J., and Craig, E. A. (1990) *J. Bacteriol.* **172**, 2055–2064
34. Jones, E. W. (1991) *Methods Enzymol.* **194**, 428–453