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

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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 support growth in the absence of the essential wild-type protein. K108A/R213A Mge1 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

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[1] The abbreviations used are: Hsp70, 70-kDa class heat shock protein; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

TABLE I
Bacterial and yeast strains

Strain	Genotype and phenotype	Source or Ref.
E. coli		
PK101	MG1655, F^-,*dnaK14 dnaJ15*	33
OD212	AM267, *dnaK332* Δ*grpE*::omega-cam^R	17
DA259	C600, *thr*::Tn*10 grpE*Δ::omega-cam^R kan^R	28
DA810	B178, *pheA*::Tn*10 grpE280*	27
RLM569	C600, *hsdR leu-pro-lac-tonA rpsL (str^R) recA*	22
S. cerevisiae		
BJ3497	*pep4:HIS3 ura3–52 his3Δ200*	34
JD100	*lys2 ura3–52 Δtrp1 leu2–3, 112 ssc1–1(LEU2)*	11
BM37–7	*trp1–1 ura3–1 leu2–3,112 his3–11,15 ade2–1 can1–100 GAL2$^+$ met2-Δ1 lys2-Δ2 SSC1ΔClaI::LEU2 mge1–2 [pRS316K-SSC1-MGE1]*	This study

the stabilization of the interaction between the two proteins but is not critical in the facilitation of nucleotide release.

EXPERIMENTAL PROCEDURES

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, pWSK29ΔS.E, was constructed by cleaving pBW401 with *Sal*I and *Eco*RI 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 pWSK29*grpE*K82A, pWSK29*grpE*R183A, and pWSK29*grpE*K82A/R183A. pRLM232 harbors the *dnaJ* gene under the control of the λ p_L and p_R promoters, both of which are controlled by the thermosensitive λ *c*I repressor encoded by the plasmid (22).

S. cerevisiae Plasmids—The plasmids used in the purification of Mge1, pGEX-KT-MGE1, and the GST-Ssc1 fusion, pRD56CS-SSC1, 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-SSC1-MGE1 was generated from pRS314-SSC1 (11).

Site-directed Mutagenesis

Mutations K108A, R213A, and K108A/R213A in *MGE1* and K82A, R183A, and K82A/R183A in *grpE* were generated by PCR by using a standard two-step PCR procedure (23). Primers used to generate the mutants are as follows: K82A, 5′-CTGGATATTGAAGCTGCCCACAAAT-3′; K108A, 5′-AGGATATTCAGAAAGCTAAGGACT-3′; R183A, 5′GCTAAATGGTGCTACGATTCGTG-3′; R213A, 5′-TCACCTTGAATGACNNNGTTATCAGACCAGCAAAAGTC-3′. The mutated fragments were cloned into appropriate plasmids and sequenced to verify the presence of the desired mutation and the absence of other mutations.

Testing of in Vivo Phenotypes

To test for the ability of mutant Mge1 proteins to rescue the inviability of strains lacking Mge1, BM37-7 carrying plasmid pRS316-SSC1-MGE1 was transformed with various pRS314-SSC1-MGE1 plasmids that carry the wild-type *MGE1* gene or mutant variants. Transformants were selected on minimal media lacking tryptophan; colonies were then patched onto the same medium containing 5-fluoroorotic acid (Toronto Research Chemicals, Inc.) to select for cells having lost the pRS316 plasmid containing the *URA3* gene and wild-type *SSC1* and *MGE1* genes but retaining the pRS314 plasmid containing the *TRP1*, wild-type *SSC1,* and mutant *MGE1* genes. Cells were tested on YPD plates at 30 and 37 °C.

E. coli strains OD212, DA259, and DA810 were grown in L broth supplemented with appropriate antibiotics and transformed with pWSK29ΔSE and variants containing mutant *grpE* genes or lacking a *grpE* gene. Transformants were selected by plating on medium containing appropriate antibiotics. Growth of transformants was tested by plating serial dilutions of cultures onto plates containing L broth supplemented with the appropriate antibiotics; growth was observed after overnight incubation at various temperatures.

Protein Expression and Purification

Mutant and wild-type Mge1 were purified essentially as described previously (11). Briefly, *E. coli* PK101 cells harboring the expression plasmid pGEX-KT-MGE1 were grown at 30 °C and induced for expressed by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. 3 h after addition of 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.

Ssc1 was purified from yeast strain BJ3497 carrying the expression plasmid pRD56CS-SSC1 which expresses a GST-SSC1 fusion. The purification procedure was essentially the same as that used for preparation of Mge1 (11), except Ssc1 was further purified using DEAE-Sepharose chromatography after cleavage with thrombin. DnaJ expression was induced in RLM569 carrying the plasmid pRLM232 as described by Karzai and McMacken (22). DnaJ was purified as described in Zylicz *et al.* (24) using DEAE-Sephacel, followed by ammonium sulfate precipitation and hydroxylapatite chromatography. DnaK was purifed as described by Kamath-Loeb *et al.* (25), using a DEAE-Sepharose column, followed by ATP-agarose and hydroxylapatite chromatography.

Luciferase Refolding Assay

1 μl of a 1 mg/ml solution of luciferase (Sigma) dissolved in 1 M glycylglycine was added to 5.4 μl of unfolding buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 6 M 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 $MgCl_2$, 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 reactions were rapidly diluted 1:25 with dilution buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 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-SSC1-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 $MgCl_2$, 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) crosslinked with purified anti-Ssc1 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 Ssc1. ECL Western blots (Amersham Pharmacia Biotech) were performed according to manufacturer's suggestions.

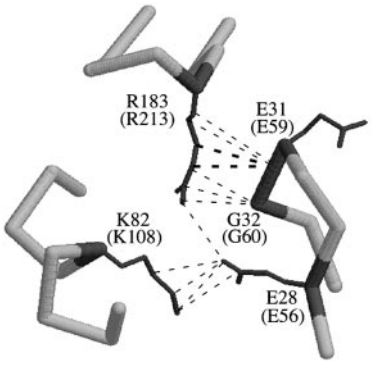

FIG. 1. **Interaction of GrpE with the loop of DnaK from amino acids 28 to 34.** Individual interactions between GrpE (*on the left*) and DnaK (*on the right*) are indicated by the *dashed lines*. *Heavy dashed lines* indicate two interactions, one with the oxygen and one with the carbon of the carbonyl group of Glu-31. Designations in *parentheses* indicate the analogous residues in Ssc1 and Mge1. Information for figure taken from Harrison *et al.* (15).

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 7.4), 50 mM KCl, 10% glycerol, 1 mM EDTA), an equal volume of 0.1 μM Mge1 was added to the beads and incubated at 4 °C for 1 h. The beads were then sequentially washed with buffer D, buffer D containing 1 M KCl, buffer D and eluted with buffer D containing 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.

Single Turnover ATPase Assay

Complex formation and single turnover assay 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-^{32}P]ATP (DuPont, 3000 Ci/mmol) in buffer E (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 56–62 of Ssc1 was previously implicated in the nucleotide release activity of Mge1 (11). Mutant proteins with certain single amino acid substitutions such as G60D were able to bind Mge1, but nucleotide release was not observed. We reasoned that if the G60D mutation disrupted a critical interaction with Mge1, it might be possible to partially overcome this defect by alterations in Mge1 that could promote nucleotide release in the Mge1-G60D Ssc1 complex. Therefore we attempted to isolate suppressor mutations in *MGE1* which rescued the lethality of the G60D mutant. Two strategies were used. *MGE1* was randomly mutagenized over its entire length using error-prone PCR. In addition, codon Arg-213 was targeted for mutagenesis because the side chain of this highly conserved encoded arginine is predicted to interact with Gly-60 (Fig. 1) (15). Oligonucleotides that could encode each possible amino acid at codon 213 were used to create a library of *MGE1* genes containing alterations at this residue. No suppressors were identified by either method (data not shown).

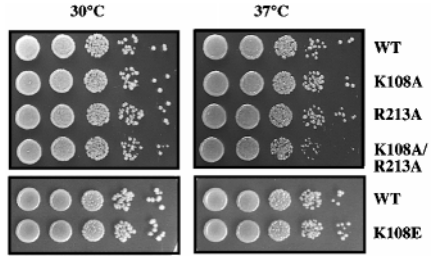

FIG. 2. **Growth phenotype of *MGE1* mutants.** Serial dilutions of strain BM37-7 carrying wild-type (*WT*) *MGE1* or mutant *MGE1* genes were plated on rich media and incubated for 2 days at 30 and 37 °C as indicated.

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 a *URA3*-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-

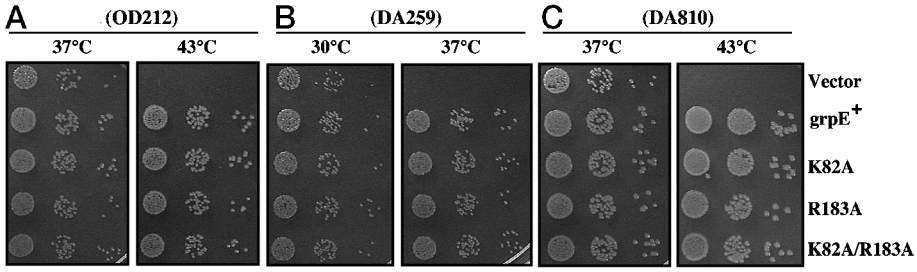

FIG. 3. **Phenotype of *grpE* mutants.** Serial dilutions of strains carrying the control plasmid pWSK29Δ (*vector*), wild-type grpE (*grpE*$^+$), or *grpE* point mutants (*K82A, R183A,* or *K82A/R183A*) were plated onto rich media and incubated overnight at the indicated temperatures. *A,* strain OD212, a *grpE* deletion strain carrying a *dnaK* allele which is viable in the absence of GrpE. *B,* strain DA259 carrying an uncharacterized extragenic suppressor of the *grpE* deletion. *C,* strain DA810 carrying a temperature-sensitive *grpE* mutation.

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 *grpE*. As can be seen in Fig. 3 all the mutants restored growth of the test strains to that observed in the presence of wild-type *grpE*. Therefore, Lys-82 and Arg-183 of GrpE, 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. 4*A*). 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. 4*B*). 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.

On the other hand, no effect on ATP hydrolysis was observed upon addition of 8 μM K108A/R213A Mge1 (Fig. 4*C*). However, addition of higher amounts of mutant Mge1 resulted in a reduction in hydrolysis. For example, a 24% reduction in hydrolysis occurred upon addition of K108A/R213A at a concentration of 24 μM at 10 min of incubation. These results suggest that K108A/R213A does interact with Ssc1 and can effect nucleotide release, but that mutant protein is less effective than 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 Luciferase Refolding Activity—To assess the ability of mutant Mge1 to function in a more complex biological assay, we set up a luciferase refolding assay utilizing Mge1 as the nucleotide exchange factor. Since Mge1 has been shown to act as a release factor for DnaK (16), we utilized DnaK, DnaJ, and Mge1 for these assays. First, conditions were established under which refolding of luciferase after chemical denaturation was dependent upon the addition of Mge1 (Fig. 5*A*). Under conditions where the ratio of DnaK:DnaJ was 1:1, addition of Mge1 increased the folding of luciferase about 6-fold, whereas at lower DnaJ concentrations, such as a 20:1 ratio, a negligible increase in refolding was found upon addition of Mge1 to the reaction.

Using equimolar amounts of DnaK and DnaJ, the ability of a variety of concentrations of wild-type Mge1 and K108A/R213A Mge1 to stimulate refolding of luciferase was tested (Fig. 5, *B* and *C*). K108A/R213A Mge1 was able to stimulate luciferase refolding but was not as effective as wild-type protein. For example, while addition of wild-type Mge1 to a concentration of 0.4 μM increased the yield of active luciferase 5-fold, addition of the mutant increased the yield less than 2-fold. However, addition of K108A/R213A Mge1 to a concentration of between 1.6 and 3.2 μM allowed similar rates of refolding as 0.4 μM wild-type Mge1. Therefore, Mge1 was able to function in the refolding assay, but about a 10-fold higher level of mutant protein, compared with wild-type protein, was required to attain the same level of activity.

Effect of Alterations of Mge1 Residues Lys-108 and Arg-213 on Interaction with Ssc1—The results of the single turnover ATPase and luciferase assays indicated that K108A/R213A Mge1 is able to function as a release factor but less effectively than wild-type. The decreased activity could be the result of a mutant protein that binds Ssc1 stably but is less effective in stimulating release, or a protein that binds much less well than wild-type protein but is as effective as wild-type in stimulating release. To try to determine the cause of the decreased activity, we assessed the effect of alanine substitutions at positions Lys-108 and Arg-213 of Mge1 on the physical interaction between the two proteins. We purified the mutant proteins and tested their interaction with Ssc1. Ssc1 was expressed as a glutathione *S*-transferase (GST) fusion and bound to glutathi-

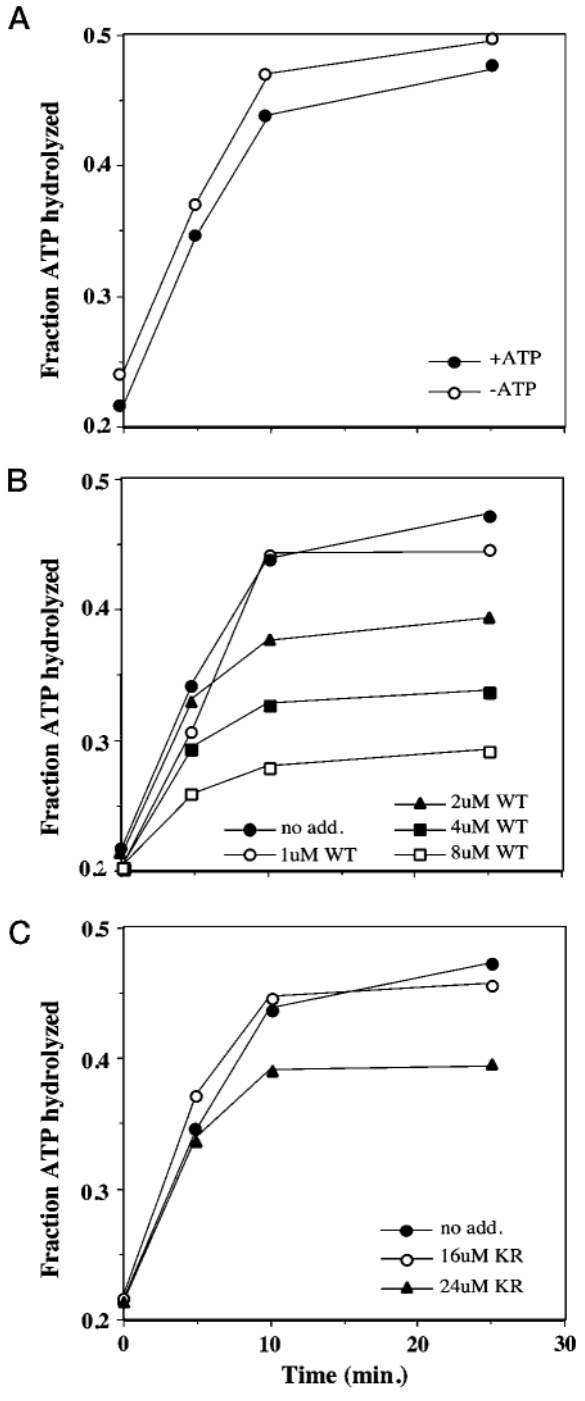

FIG. 4. **Effects of *MGE1* mutations on the single turnover of Ssc1-ATP complex.** Ssc1-ATP complex (~2 μM) was incubated at 30 °C in the presence or absence of 250 μM ATP and various concentrations of wild-type (*WT*) and mutant Mge1. Samples were withdrawn at the indicated times and the fraction of ATP converted to ADP determined. *A,* hydrolysis of ATP in the absence of Mge1. *Open circles*, no ATP added; *closed circles,* ATP added. *B,* hydrolysis of ATP in the presence of ATP and different concentrations of wild-type Mge1, as indicated. *C,* hydrolysis of ATP in the presence of ATP and different concentrations of K108A/R213A Mge1.

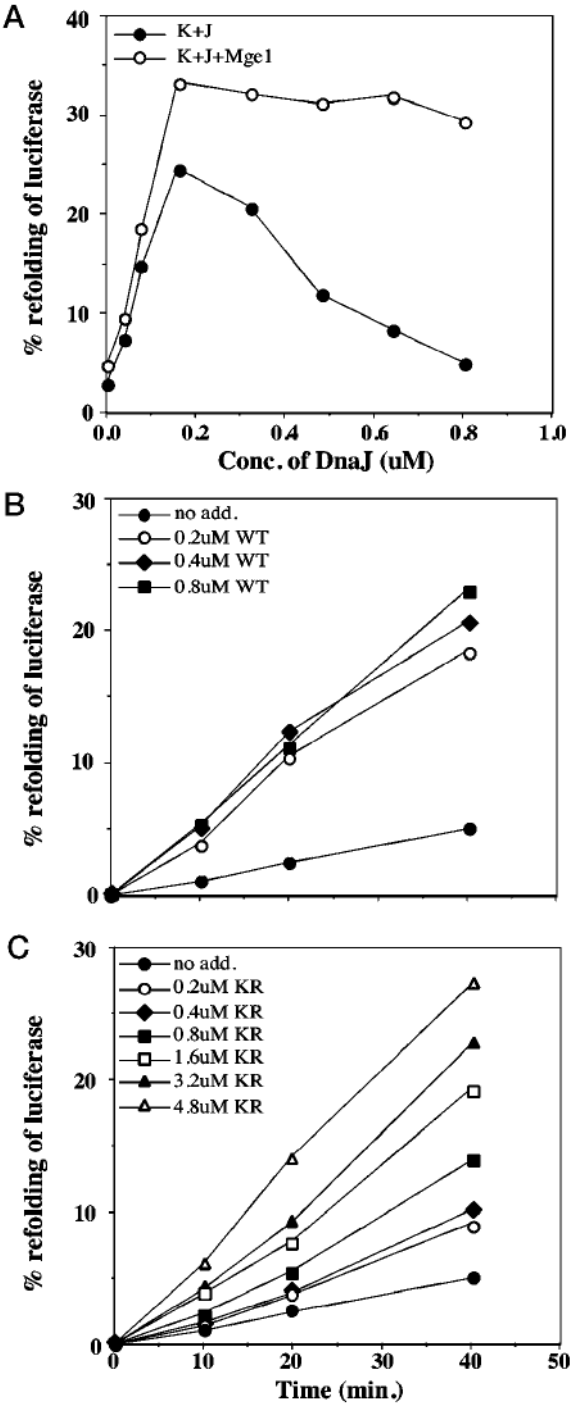

FIG. 5. **The dependence of refolding on the presence of Mge1.** Luciferase was denatured in guanidine HCl and diluted into buffer containing 0.8 μM DnaK and various concentrations (*Conc.*) of DnaJ and wild-type or mutant Mge1. Refolding was monitored by measuring enzymatic activity in a luminometer as described under "Experimental Procedures." *A,* refolding assays were carried out in the presence of 0.8 μM DnaK and either the absence (*closed circles*) or 0.8 μM (*open circles*) Mge1 and concentrations of DnaJ up to 0.8 μM for 20 min. *B,* refolding of luciferase in the presence of wild-type Mge1 and 0.8 μM DnaK and DnaJ. Refolding assays were done in the absence (*closed circles*) of Mge1 or in the presence of the indicated amounts of wild-type Mge1. *C,* refolding of luciferase in the presence of K108A/R213A Mge1. Refolding assays were done in the absence (*closed circles*) of Mge1 or in the presence of the indicated amounts of K108A/R213A Mge1.

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

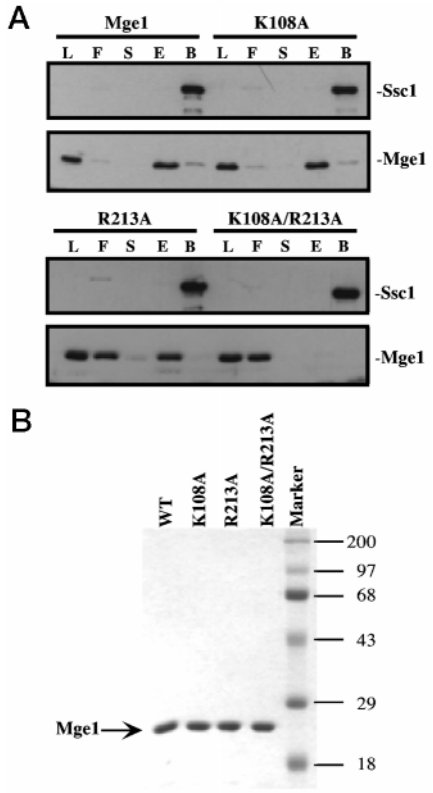

FIG. 6. **Effect of *mge1* amino acid substitutions on binding to Ssc1.** *A,* GST-Ssc1 was immobilized on glutathione-agarose beads. Purified wild-type (Mge1) or mutant proteins (*K108A, R213A,* and *K108A/R213A*) were mixed with the beads which were then extensively washed and eluted with 1 mM ATP. *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.

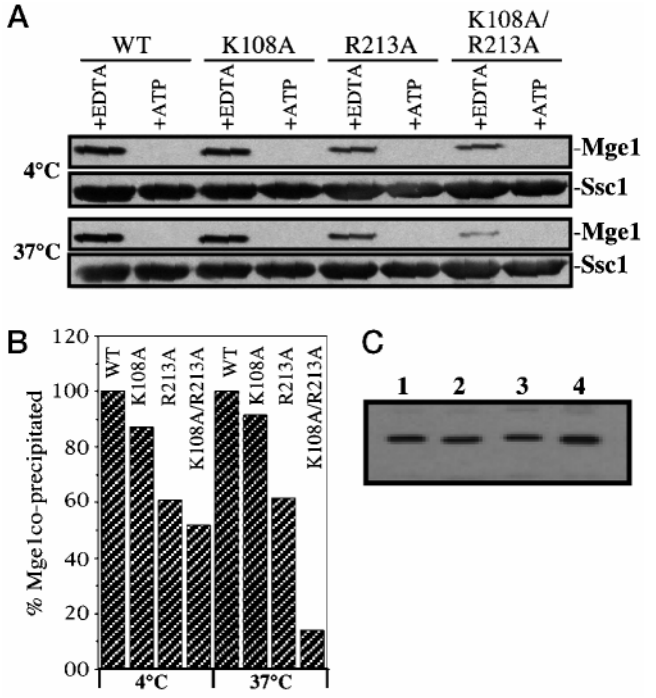

FIG. 7. **Co-immunoprecipitation of Mge1 with Ssc1 in mitochondria.** *A,* isolated mitochondria were disrupted and subjected to immunoprecipitation with Ssc1-specific antibody in the presence of either ATP and Mg^{2+} (*+ATP*) or EDTA (*+EDTA*). The precipitates were resuspended and aliquots separated by SDS-PAGE. The gels were subjected to immunoblot analysis using Mge1 or Ssc1–specific antibody as indicated. *B,* the signals in *A* (*+EDTA* only) using Mge1-specific antibody were quantitated by densitometry; wild-type was set at 100%. *C,* aliquots of the lysed mitochondria were directly subjected to immunoblot 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).

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 *in vivo*. Mitochondria from strains carrying wild-type or mutant *MGE1* genes were incubated at 4, 30, or 37 °C for 10 min and then disrupted with lysis buffer which contained Triton X-100. The resulting lysate was subjected to immunoprecipitation with Ssc1-specific antibodies. Immunoblot analysis was carried out on the precipitate to determine the amount of Ssc1 precipitated. Similar amounts of Ssc1 were found in all the precipitates indicating similar efficiencies of precipitation with the antibody. In addition, similar amounts of Mge1 protein was present in wild-type and mutant extracts (Fig. 7*C*).

Mge1 antibody was used for immunoblots to determine whether Mge1 was co-precipitated with Ssc1. In the presence of added ATP, no co-immunoprecipitation of Mge1 was observed in any sample, as expected. When EDTA was added to the lysis buffer, wild-type Mge1 and K108A were present in the immunoprecipitates in approximately equal amounts regardless of the incubation temperature (Fig. 7, *A* and *B*). However, less R213A and K108A/R213A Mge1 than wild-type Mge1 was co-immunoprecipitated from mitochondria maintained at 4 °C. Similar results were obtained when mitochondria were preincubated at 30 °C prior to immunoprecipitation (data not shown). This reduction in interaction of K108A/R213A Mge1 was more pronounced when the precipitation was done with mitochondria preincubated at 37 °C, as only about 14% as much Mge1 was co-immunoprecipitated with Ssc1 antibody in 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 Ag-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 interaction 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 Å^2. 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 organellar* 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 effect 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 K_d 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.

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