Compensation for a Defective Interaction of the Hsp70 Ssq1 with the Mitochondrial Fe-S Cluster Scaffold Isu*

Helena Knieszner‡§, Brenda Schilke§¶, Rafal Dutkiewicz‡, Patrick D'Silva¶, Sara Cheng¶, Maikke Ohlson¶‖, Elizabeth A. Craig¶**, and Jaroslaw Marszalek‡¶

From the ‡Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdansk, 24 Kladki, 80-822 Gdansk, Poland and ¶Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Ssq1, a specialized yeast mitochondrial Hsp70, plays a critical role in the biogenesis of proteins containing Fe-S clusters through its interaction with Isu, the scaffold on which clusters are built. Two substitutions within the Ssq1 substrate binding cleft, both of which severely reduced affinity for Isu, had very different effects *in vivo*. Cells expressing Ssq1(F462S), which had no detectable affinity for Isu, are indistinguishable from Δ*ssq1* cells, underscoring the importance of the Ssq1-Isu1 interaction *in vivo*. In contrast, cells expressing Ssq1(V472F), whose affinity for Isu is at least 10-fold lower than that of wild-type Ssq1, had only moderately reduced Fe-S enzyme activities and increased iron levels and grew similarly to wild-type cells. Consistent with the reduced affinity for Isu, the ATPase activity of Ssq1(V472F) was stimulated less well than that of Ssq1 upon addition of Isu and Jac1, the J-protein partner of Ssq1. However, higher concentrations of Jac1 or Isu1, which form a stable complex, could compensate for this defect in stimulation of Ssq1(V472F). Expression of Isu1 was up-regulated 10-fold in *ssq1(V472F)* compared with wild-type cells, suggesting that formation of a Jac1-Isu1 complex can overcome a lowered affinity of Ssq1 for Isu *in vivo* as well as *in vitro*.

Chaperones of the Hsp70 family function in a number of essential cellular roles, including *de novo* protein folding, refolding of unfolded proteins, and protein translocation across intracellular membranes (1–3). These various activities rely on the ability of Hsp70s to reversibly bind hydrophobic segments of proteins. Affinity of Hsp70s for substrate depends on the conformation of the C-terminal substrate binding domain, which is regulated by the nucleotide bound to the N-terminal ATPase domain. When ATP is bound to Hsp70, an open conformation of the substrate binding domain allows fast binding and release; when ADP is bound, a closed conformation of the substrate binding domain results in slow binding and release. Under physiological conditions, when ATP concentrations are typically high substrate protein interacts with Hsp70 in the ATP conformation. This interaction is stabilized upon hydrolysis of ATP. The cycle of interaction is completed when ADP is replaced by ATP and the substrate is released.

Thus, stimulation of the ATPase activity of Hsp70 is essential for stabilization of the Hsp70-substrate interaction. However, the rate of the intrinsic ATPase activity of Hsp70 is low. ATPase activity is stimulated both by interaction of the substrate in the substrate binding cleft and J-protein co-chaperone interaction with the ATPase domain. Some J-proteins not only stimulate the ATPase activity of their partner Hsp70 but also bind substrates directly. This interaction has led to the suggestion that these proteins play an important role in targeting substrates for Hsp70 binding (4–7).

Biogenesis of Fe-S clusters is a critical step in the maturation of the numerous cellular proteins that contain this prosthetic group. In yeast mitochondria, a specialized Hsp70, Ssq1, plays an important role in this process. It interacts with the scaffold protein, Isu, on which an Fe-S cluster is built prior to transfer to a recipient apoprotein (for review see Refs. 8–10). Most members of the Hsp70 protein family bind short hydrophobic peptide sequences with relatively low specificity (1, 2). In contrast, Hsp70s specialized in biogenesis of Fe-S centers, both in bacteria (Hsc66) and yeast mitochondria (Ssq1), display high substrate specificity, interacting with the unique PVK tripeptide within the IscU/Isu protein (11–16) that is located in an exposed loop between two α-helices of the folded protein (17).

Ssq1 functions with the specialized J-protein partner Jac1. As expected of a J-protein partner, Jac1 specifically stimulates the ATPase activity of Ssq1 (13) and promotes formation of stable Ssq1-Isu complex in the presence of ATP. In addition, alterations within the signature HPD motif of the J-domain result in a decrease of *in vitro* activity and have profound phenotypic effects *in vivo* (13, 18, 19). The role of Jac1 in Ssq1-Isu interaction is not limited, however, to the stimulation of ATPase activity, as Jac1 binds Isu1 (11, 13). Thus the Ssq1-Jac1 chaperone pair shares many features with other chaperone systems but is very unusual in that it is thought to have a single specific substrate protein. Most Hsp70s interact with many amino acid segments of hydrophobic character in many partially folded proteins. Previously, we found that alterations in the PVK tripeptide of Isu led to disruption of the interaction with Ssq1 and deleterious *in vivo* phenotypes (12). However, these preliminary results suggested that the cell could tolerate alterations that decreased the affinity for Ssq1 in *in vitro* assays to a surprising degree.

Therefore, we set out to analyze the effect of alterations in

§ Both authors contributed equally to this work.

‖ Present address: Dept. of Microbiology, University of Washington, Seattle, WA 98195.

** To whom correspondence should be addressed: Dept. of Biochemistry, 441E Biochemistry Addition, 433 Babcock Dr., University of Wisconsin, Madison WI 53706-1544. Tel.: 608-263-7105; Fax: 608-262-3453; E-mail: ecraig@wisc.edu.

the substrate binding cleft of Ssq1. A substitution that decreased the affinity for Isu more than 10-fold had only moderate effects *in vivo*. Our results suggest that up-regulation of Isu expression, coupled with highly specific interactions between it and the chaperones Ssq1 and Jac1, can compensate for a low affinity of Ssq1 for Isu.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Media, and Chemicals—The *ssq1(V472F)* and *ssq1(F462S)* alleles were constructed by site-directed PCR mutagenesis and cloned into pRS306 (20). These alleles were integrated into the chromosome using the two-step protocol. The vector was linearized using the XhoI site found in the 5′-untranslated region of *SSQ1* or using the NcoI site found in the *URA3* gene and transformed into a diploid strain heterozygous for Δ*ssq1::LYS2* (21) using the lithium acetate protocol (22). Ura$^+$ transformants were first patched onto YPD medium and then onto medium containing 5-FOA to identify candidates that had lost the vector sequence by homologous recombination when integration was directed to the *SSQ1* locus. Cells that grew on 5-FOA were tested for growth on minimal medium lacking lysine, which is indicative of replacement of the *SSQ1* gene deletion with the mutant copy of the gene. Ura$^-$ Lys2$^-$ diploids were sporulated and haploids obtained by dissection. When integration was directed to the *SSQ1* locus, Ura$^+$Lys$^+$ transformants were sporulated and Ura$^+$Lys$^+$ haploids obtained by dissection. The presence of the *ssq1(V472F)* and *ssq1(F462S)* alleles was determined by sequencing the *SSQ1* PCR products generated from chromosomal DNA isolated from the haploid strains. Integration of a mutant *SSQ1* allele at the *SSQ1* or *URA3* locus resulted in similar phenotypes.

To assess genetic interactions between *ssq1(V472F)* and *isu1* mutants, the *ssq1(V472F)* strain was crossed to a Δ*isu1*,Δ*isu2* strain carrying a wild-type copy of *ISU1* on a *URA3*-marked plasmid. After sporulation and dissection, the haploid strain, *ssq1(V472F)* Δ*isu1*,Δ*isu2* with pRS316-*ISU1*, was transformed with a *TRP1*-marked vector carrying mutant alleles of *ISU1*. The ability of the strains to grow in the absence of wild-type Isu1 was tested by streaking onto 5-FOA medium.

Yeast were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) or on synthetic medium prepared as described (23). All chemicals, unless stated otherwise, were purchased from Sigma.

Mitochondrial Iron and Respiratory Enzyme Measurements—Mitochondrial iron levels were determined as described (18). Activities of the respiratory enzymes were measured in isolated mitochondria. Succinate dehydrogenase activity was measured by using succinate as a substrate as described (24), except 2,6-dichloroindophenol was used in place of cytochrome *c* (25). Aconitase activity was measured by monitoring the decrease in absorbance of the substrate isocitrate at 240 nm as described (26, 27). Data were normalized to the protein content of the mitochondrial samples.

Purification of Proteins—Recombinant Jac1$_{His}$ (18), Mge1$_{His}$ and Mdj1$_{His}$ (28), Ssq1$_{His}$ and Isu$_{His}$ wild-type and mutant proteins (13) were purified as previously described. The C terminus of *SSQ1*-(424–657), mutant V472F-(424–657), and F462S-(424–657) were cloned into the *Escherichia coli* plasmid pET3a. Wild-type and mutant protein expression was induced at 30 °C by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The *E. coli* cell pellets were resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 250 mM KCl, 10% glycerol, 1 mM dithiothreitol, 10 mM imidazole, EDTA-free protease inhibitors (Roche Applied Science), 0.05% Triton X-100, and subjected to treatment in a French Press twice at 4 °C. Extracts were clarified by 12,000 × *g* centrifugation for 30 min at 4 °C. The clear supernatant was subjected to affinity chromatography using nickel-nitrilotriacetic acid-agarose. The column was washed with 10 volumes of buffer A, two volumes of buffer B (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 60 mM imidazole, 5 mM ATP), two volumes of buffer C (20 mM Tris-HCl, pH 8.0, 1 M KCl, 10% glycerol, 60 mM imidazole), and two volumes of buffer D (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 60 mM imidazole). Bound proteins were eluted with buffer E (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 500 mM imidazole) and dialyzed against buffer F, (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM magnesium acetate, 10% glycerol).

Protein concentrations, determined using the Bradford (Bio-Rad) assay system using bovine serum albumin as a standard, are expressed as the concentration of monomers. All wild-type His-tagged proteins used in this study were able to functionally replace untagged protein. Functionality was tested by constructing strains in which the only copy of a gene encoding a particular protein was a His-tagged version harbored on the low copy plasmid, pRS316 (20). Growth of such strains was indistinguishable from their wild-type derivatives in medium containing different carbon sources at different temperatures (data not shown).

Surface Plasmon Resonance (SPR) Analysis—SPR^1 studies were carried out at 25 °C with a Biacore 2000 instrument (Piscataway, NJ). Peptide P-**P**VK (LSLP**P**VKLHC) was cross-linked to the surface of the sensor chip CM5 by thiol coupling as recommended by the manufacturer. Purified protein Isu1 was randomly cross-linked to the surface of the sensor chip CM5 by amine coupling as recommended by the manufacturer. Binding experiments were conducted in buffer R (25 mM HEPES-KOH, pH 7.5, 200 mM KCl, 11 mM MgCl$_2$, 0.005% (v/v) surfactant P20; Amersham Biosciences) containing 2 mM ATP or 2 mM ADP when indicated with the running buffer at a flow rate of 10 μl/min. 60 μl of buffer R containing indicated purified proteins (Jac1, Ssq1, Ssq1(V472F)) was used for injections.

Fluorescence Anisotropy Measurements—Isu peptides LSLP-**P**VKLHC and LSLP**A**VKLHC were labeled at the C terminus with fluorescein to generate P-**P**VK-F* and P-**A**VK-F* as described (29). Various concentrations of either wild-type Ssq1-C terminus, V472F-C terminus, or F462S-C terminus proteins were incubated with P-**P**VK-F* and P-**A**VK-F* (10 nM) at 25 °C in buffer F for 45 min. After binding reached equilibrium, anisotropy measurements were made with the Beacon 2000 fluorescence polarization system (Panvera, Madison, WI) at 25 °C with excitation at 490 nm and emission at 535 nm. The data were fitted to one-site hyperbolic equation using Prism (GraphPad).

Circular Dichroism and Partial Proteolysis—Measurements were performed on an Aviv 62A DS Circular Dichroism spectrometer from 196 to 260 nm with 5-s averaging times and 1-nm step size at 25 °C. The protein concentration was 5 μM in 10 mM Tris-HCl, pH 7.5, 80 mM KCl, in a quartz cuvette with 1-mm path length. Spectra were measured in millidegrees, corrected for buffer effects, and converted to mean residue ellipticity [Θ].

Proteolytic cleavage of Ssq1 and Ssq1(V472F) was carried out at 25 °C by incubating protein (4 μg) with trypsin (0.03 μg) in 40 μl of buffer consisting of 40 mM HEPES-KOH, pH 7.6, 8 mM magnesium acetate, 0.3 mM EDTA, 2 mM dithiothreitol, 20 mM NaCl, 20 mM KCl, and 2 mM ATP. At specific times, samples were taken, and the reaction was terminated by adding 20 μl of SDS sample buffer. The samples were immediately boiled for 3 min.

Other Techniques—Glycerol gradient centrifugation and steady-state ATPase assays were carried out as described previously (13). In the ATP assays release of radioactive inorganic phosphate from [γ-^{32}P]ATP was measured. Control reactions lacking protein were included in all experiments.

RESULTS

Phenotypic Effects of Alterations in the Substrate Binding Cleft of Ssq1—To investigate the functional importance of the interaction of Ssq1 with its protein substrate, Isu1, we introduced alterations in Ssq1 at residues analogous to those previously shown to be important for the ability of other Hsp70s to bind substrate proteins (29–31). Valine 472 was replaced by phenylalanine, and phenylalanine 462 was replaced by serine to generate Ssq1(V472F) and Ssq1(F462S), respectively. Yeast cells expressing Ssq1(F462S) grew indistinguishably from *SSQ1* deletion cells (Fig. 1*A* and data not shown). In addition, compared with wild-type mitochondria, levels of iron were 4-fold higher and the activity of the Fe-S cluster containing enzymes aconitase and succinate dehydrogenase was >10-fold lower in *ssq1(F462S)* mitochondria, similar to effects seen in Δ*ssq1* mitochondria (Fig. 1, *B* and *C*). The similarity in phenotype caused by the complete lack of Ssq1 and a single amino acid alteration in the peptide binding cleft is consistent with the proposed importance of substrate binding ability of Ssq1 for its *in vivo* function.

On the other hand, cells harboring *ssq1(V472F)* as the only copy of *SSQ1* were able to grow nearly as well as wild-type cells (Fig. 1*A*, and results not shown). However, *ssq1(V472F)* mitochondria did accumulate 2-fold more iron than did wild-type mitochondria, indicating that iron homeostasis was affected to some degree (Fig. 1*B*). Moreover, activities of enzymes contain-
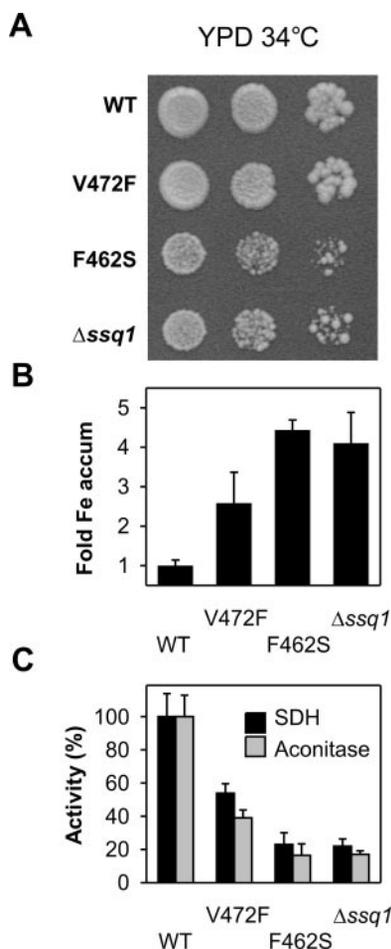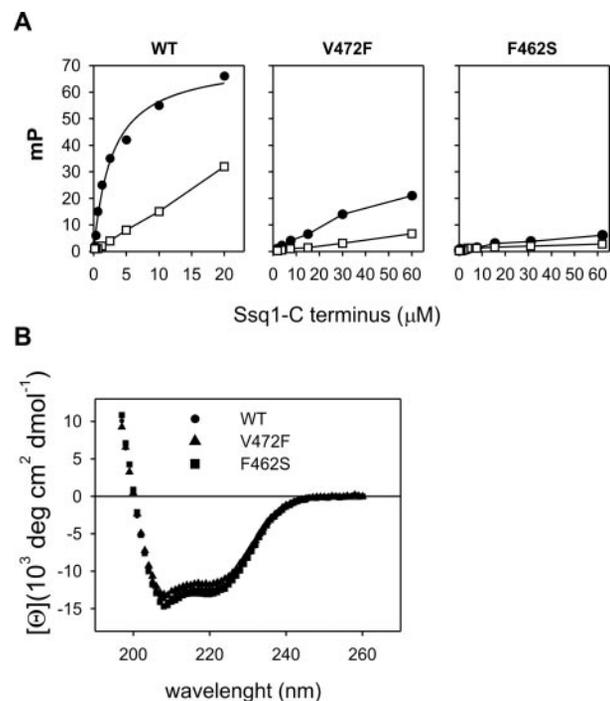

FIG. 1. **Phenotypes of cells harboring *ssq1(V472F)* and *ssq1(F462S)*.** *A*, cells harboring a chromosomal copy of *ssq1(V472F)* and *ssq1(F462S)* were plated on glucose-rich medium. Plates were incubated at 34 °C for 3 days. Iron levels (*B*) and enzyme activities (*C*) in lysates of mitochondria isolated from *ssq1(V472F)* and *ssq1(F462S)* cells grown in low iron galactose minimal medium. Succinate dehydrogenase and aconitase activities determined in wild-type mitochondrial extracts were set at 100%. *Bars* represent average values measured for 3–4 separate mitochondrial preparations. *Error bars* represent S.D. of measurements. Iron levels and enzyme activities values determined for mutant *ssq1(V472F)* were significantly different (paired *t* test; $p < 0.005$ and $p < 0.001$, respectively) from values obtained for wild-type and Δ*ssq1*.


FIG. 2. **Binding of C terminus of Ssq1 WT, Ssq1(V472F), and Ssq1(F462S) to peptide substrate.** *A*, fluorescein-labeled peptides P-**P**VK-F* (*circles*) or P-**A**VK-F* (*squares*) at concentrations of 10 nM were incubated in the presence of indicated concentrations of purified C terminus of wild-type Ssq1, Ssq1(V472F), or Ssq1(F462S). Fluorescence anisotropy measurements were taken. After subtraction of background polarization, minipolarization (*mP*) was plotted. *B*, CD spectra measured for purified C terminus of Ssq1(WT), Ssq1(V472F), and Ssq1(F462S) as described under "Experimental Procedures."

ing Fe-S centers were reduced in mitochondria isolated from *ssq1(V472F)*: aconitase activity to 39% and succinate dehydrogenase to 54% of wild-type levels (Fig. 1*C*). The mild phenotypic effects of *ssq1(V472F)* were unexpected because homologous substitutions in DnaK and another mitochondrial Hsp70, Ssc1 (V436F and V459F, respectively) resulted in null phenotypes (30) (data not shown).

Both Ssq1 (V472F) and (F462S) Have Reduced Affinity for Substrate—Because of the unexpected phenotypic differences between *ssq1*(V472F*)* and *ssq1*(F462S*)*, we investigated the biochemical properties of the two mutant proteins. First, we utilized fluorescence anisotropy to measure substrate binding. The C-terminal 233-amino acid fragment, which contains the peptide binding domain, was utilized because of technical difficulties in obtaining large quantities of full-length Ssq1.

The peptide LSLP**P**VKLHC labeled with fluorescein (designated P-**P**VK-F* throughout) was used as substrate. LSLP-**P**VKLHC encompasses amino acids 130–139 of the Isu1 sequence and contains the PVK tripeptide essential for interaction with Ssq1 (12). Analysis of the C terminus of wild-type Ssq1 yielded a dissociation constant (K_d) of 2.81 ± 0.40 μM

for P-**P**VK-F* (Fig. 2*A*). As a test of the specificity of this assay we utilized the fluorescein-labeled peptide LSPA**V**KLHC (P-**A**VK-F*), because Isu1 binding to Ssq1 was strongly reduced when residue Pro^{134} within the PVK motif of Isu1 was replaced by Ala (12). Binding to P-**A**VK-F* peptide was significantly reduced compared with P-**P**VK-F*.

In contrast, virtually no binding of either peptide was observed to Ssq1(F462S), indicating that this alteration within the peptide binding pocket severely interfered with substrate binding (Fig. 2*A*). The affinity of the C terminus of Ssq1(V472F) for P-**P**VK-F* peptide was also strongly reduced in comparison to the wild-type control, but changes in anisotropy were measurable. Because of practical limitations on protein concentrations, saturation was not attained. However, we estimated that the affinity of the C terminus of Ssq1(V472F) for P-**P**VK-F* was more than 10-fold reduced compared with wild-type. Moreover, the mutant C terminus displayed sequence specificity similar to the wild-type control, as binding of P-**A**VK-F* was significantly reduced in comparison to binding of P-**P**VK-F* (Fig. 2*A*). In addition, the circular dichroism (CD) spectra of wild-type and mutant Ssq1 C termini were indistinguishable, indicating that the differences in binding observed with the mutant proteins were not because of global misfolding (Fig. 2*B*).

Because of the mild phenotypic effects of the V472F substitution, even though major effects on the ability of the C-terminal domain to bind peptide were observed we decided to assess substrate binding by full-length Ssq1. Because of difficulties in obtaining large amounts of full-length protein, we turned to SPR. When ADP-bound wild-type Ssq1 was passed over the surface of a sensor chip to which P-**P**VK peptide was immobilized, a strong increase in the response signal was observed, indicating efficient binding to P-**P**VK. However, consistent with known Hsp70 biochemical properties, the maximal signal
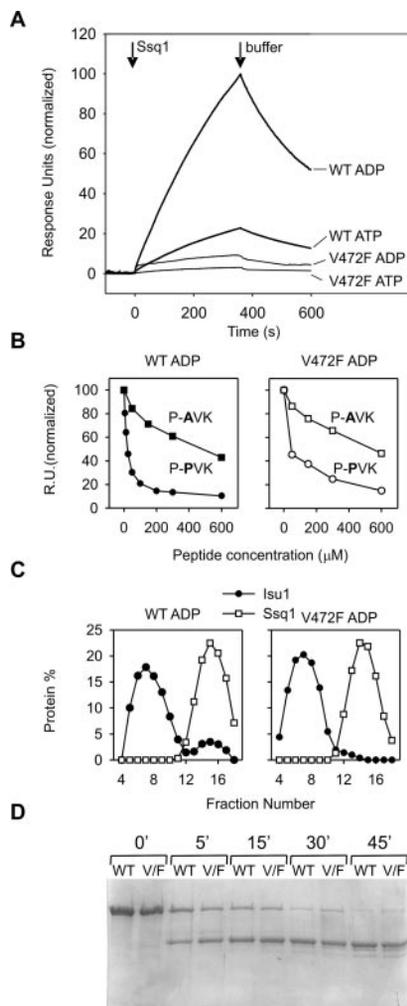
FIG. 3. **Binding of Ssq1(V472F) to peptide and protein substrates.** *A*, SPR analysis of Ssq1(WT) and Ssq1(V472F) binding to P-**P**VK in the presence of ADP (2 mM) or ATP (2 mM). P-**P**VK was cross-linked to the surface of the chip (3000 response units) via a C-terminal cysteine residue. Purified Ssq1 (1.4 μM) or Ssq1(V472F) (1.5 μM) was passed over the chip surface. *B*, substrate specificity of Ssq1WT and Ssq1(V472F). 1.2 μM Ssq1 (*left panel*) was incubated at 25 °C for 30 min, alone or in the presence of indicated concentrations of P-**P**VK or P-**A**VK. Reaction mixtures were then passed over the surface of an SPR chip having immobilized P-**P**VK (70 response units). 1.4 μM Ssq1(V472F) (*right panel*) was incubated as above with P-**P**VK or P-**A**VK. Reaction mixtures were then passed over the surface of a SPR chip with immobilized P-**P**VK (3000 response units). Maximal response units obtained for each run were normalized by setting values detected without peptide competitor as 100. *C*, Isu1 binding to Ssq1(WT) and Ssq1(V472F) was analyzed using glycerol gradient centrifugation in the presence of ADP (2 mM). Each protein was at a concentration of 5 μM during the incubation before centrifugation. *D*, partial proteolytic digestion of Ssq1-WT and Ssq1(V472F)-V/F proteins carried out for the indicated times. For details see "Experimental Procedures."

obtained for Ssq1 in the ATP state was reduced by 80% (Fig. 3*A*). In contrast, the maximal signal measured when purified Ssq1(V472F) in the ADP state was passed over the chip with immobilized P-**P**VK was at least 10-fold lower than that detected for wild-type Ssq1 (Fig. 3*A*). A further 70% reduction of maximal response units was observed when Ssq1(V472F) was injected in the ATP state.

To test whether binding of Ssq1 wild-type and mutant proteins to the immobilized P-**P**VK substrate was sequence-specific, increasing concentrations of peptide were passed over the chip together with Ssq1. As expected, binding of wild-type Ssq1 to immobilized P-**P**VK was strongly reduced in a concentration-dependent manner (Fig. 3*B*, *left panel*), with 50% reduction in

Ssq1 binding occurring at ~24 μM. On the other hand, when P-**A**VK peptide was used as a competitor, less reduction was observed, with 60% binding observed in the presence of 300 μM P-**A**VK peptide. Similarly, when Ssq1(V472F) was tested, less reduction in the binding signal was observed in the presence of P-**A**VK than in the presence of P-**P**VK (Fig. 3*B*, *right panel*). Therefore, although the Ssq1(V472F) had reduced peptide binding affinity, the residual binding appears sequence-specific.

To more closely mimic the normally occurring interaction between Ssq1 and Isu, we assessed the interaction of Ssq1(V472F) with full-length Isu1 protein, rather than peptide, using glycerol gradient centrifugation assays. Consistent with previous results (13), ~20% of the Isu1 co-fractionated with wild-type Ssq1 following centrifugation in the presence of ADP (Fig. 3*C*, *left panel*). However, no co-migration of Isu1 was observed for Ssq1(V472F) (Fig. 3*C*, *right panel*), indicating that the mutant protein did not form a stable complex with the Isu1 substrate.

We carried out limited proteolysis digestion as a means of comparing the global folding of full-length Ssq1 and Ssq1(V472F). Typical of an Hsp70, digestion of both mutant and wild-type protein proceeded over time, resulting in production of a stable 44-kDa fragment (Fig. 3*D*). Therefore, taken together, our results indicate that mutant Ssq1(V472F) was folded properly but had severely reduced affinity for both peptide and protein substrate.

Reduced Affinity of Ssq1(V472F) for Substrate Binding Is Compensated in Vitro by High Concentrations of Both Jac1 and Isu1—Because a *ssq1(V472F)* strain grows nearly as well as a wild-type strain, we hypothesized that the reduced affinity of Ssq1(V472F) for substrate must be offset by other interactions among the components of the chaperone-substrate machinery, Isu, Jac1, and Ssq1. To test this idea, we assessed functional interaction between Isu1 and Ssq1 *in vitro* by determining the ability of Isu1 to stimulate the ATPase activity of Ssq1. Efficient stimulation of the ATPase activity of Ssq1 requires the presence of the co-chaperone Jac1 and the nucleotide release factor Mge1, as well as the substrate protein Isu1. When equimolar (0.8 μM) concentrations of these proteins were used, severalfold stimulation of ATPase activity was observed (13). Under these conditions, no stimulation of Ssq1(V472F) ATPase activity was detected (data not shown), consistent with our observations that the mutant protein has a strongly reduced affinity for substrate

To investigate whether Jac1 and/or Isu1 were capable of compensating for the lower affinity of Ssq1(V472F) for substrate, we examined the stimulation of the ATPase activity of Ssq1 and Ssq1(V472F) more thoroughly. Titration of Isu1 in the presence of 20-fold excess of Jac1 over Ssq1(V472F) resulted in 3-fold stimulation of ATPase activity (Fig. 4*A*), whereas under the same experimental conditions the ATPase activity of wild-type Ssq1 was stimulated 6-fold. Therefore, high concentrations of both Jac1 and Isu1 were able to partially compensate for the low affinity of Ssq1(V472F) for substrate. However, when wild-type Isu1 protein was replaced by mutant Isu1(P134A), no stimulation of Ssq1(V472A) ATPase was observed, whereas wild-type Ssq1 ATPase was stimulated 5-fold (Fig. 4*A*). These results are consistent with the observations that Ssq1(V472F) has a lower affinity for P-**A**VK than for P-**P**VK peptide (Figs. 2*A* and 3*B*). This reduction was not unique to a single amino acid alteration, because a similar reduction in the stimulation of ATPase was observed for the combination of Ssq1(V472F) and an mutant Isu1 protein having another alteration within the PVK recognition motif, Isu1(V135A) (Fig. 4*A*).

When the reverse titration was performed using Ssq1 or
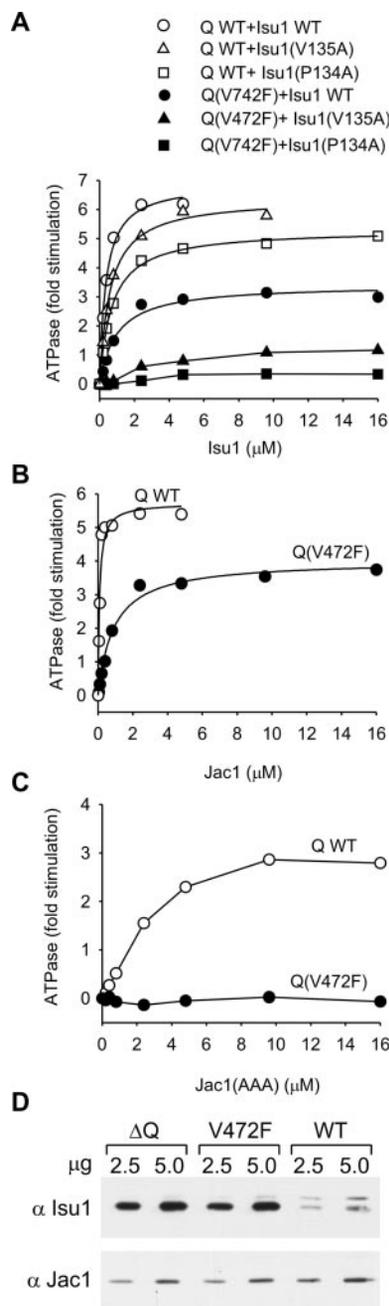
FIG. 4. **Stimulation of Ssq1(WT) and Ssq1(V472F) ATPase activity.** *A*, reaction mixtures contained 0.8 μM Ssq1 WT or V472F, 16 μM Jac1, 0.8 μM Mge1 and Isu1 WT, P134A, or V135A as indicated. ATPase activity measured in the absence of Isu1 was set to 0. *B*, as in *panel A* but with Isu1 at 16 μM, and Jac1 as indicated. ATPase activity measured in the absence of Jac1 was set to 0. *C*, as in *panel B* but with various concentrations of Jac1(AAA). *D*, protein concentration of Isu1 and Jac1 in mitochondrial extracts prepared from $\Delta ssq1, ssq1$(V472F), and *SSQ1* cells. Indicated amounts of mitochondrial extracts (μg of total proteins) were separated by SDS-PAGE. Isu1 and Jac1 were visualized by immunoblot analysis using polyclonal antibodies against each protein.

Ssq1(V472F), with Jac1 being titrated in the presence of excess Isu1, similar stimulation of ATPase activity was seen as when Isu1 was titrated (Fig. 4*B*). Because in both titration experiments a hyperbolic relationship between protein concentrations and activation of ATPase was observed, the data were fit to the Michaelis-Menten equation. This fitting allowed calculation of the concentration of proteins giving half-maximal stimulation of the ATPase activity ($C_{0.5}$), a parameter that can be taken as an approximate measure of Isu1 and Jac1 affinity for Ssq1. For wild-type Ssq1, $C_{0.5}$ values for Isu1 (0.36 ± 0.04
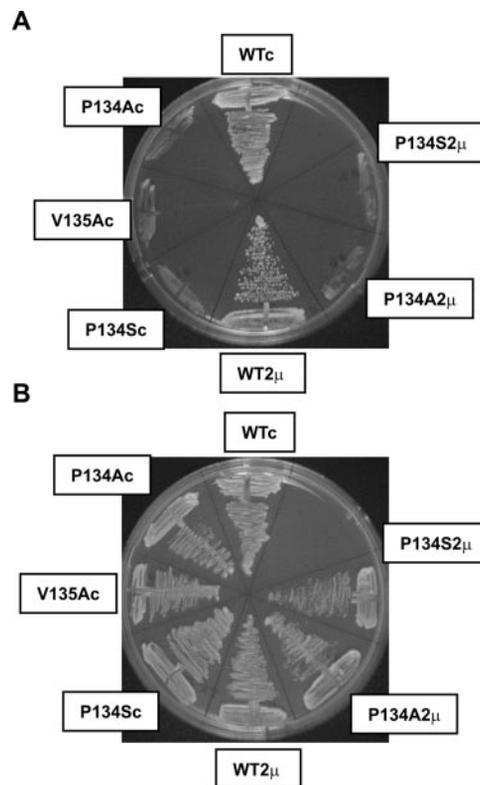
FIG. 5. **Phenotypic effects of combination of *ssq1(V472F)* with mutations in ISU1.** *A*, *ssq1(V472F)* $\Delta isu1, \Delta isu2$ cells harboring plasmid-borne copies of both wild-type *ISU1* (*URA3* marked) and mutant *isu1(P134A)*, or *isu1(V135A)*, or *isu1(P134S)* (*TRP1* marked; centromeric (*c*) or high copy (*2μ*)) plasmids) were plated on glucose-rich medium containing 5-FOA (*A*), which selects for cells having lost the plasmid that has the wild-type copy of *ISU1*, or glucose minimal medium lacking tryptophan (*B*). Plates were incubated at 30 °C for 3 days.

μM) and Jac1 (0.087 ± 0.021 μM) differed by 4-fold, indicating that both proteins could interact independently with Ssq1. In contrast, Ssq1(V472F) $C_{0.5}$ values determined for Isu1 (0.99 ± 0.18 μM) and Jac1 (0.91 ± 0.12 μM) were very similar. This similarity suggests that Jac1 and Isu1 do not bind to Ssq1(V472F) independently but instead that Jac1 binds to Isu1 first and then the Jac1-Isu1 complex interacts with Ssq1(V472F) as a single entity (12).

To test whether the J-domain of Jac1 is important in compensating for the low affinity of Ssq1(V472F) for Isu1 substrate, we used a Jac1 mutant in which three essential amino acids of the J-domain (HPD) were replaced by three alanine residues. Consistent with previous results (12) such a mutant protein Jac1(AAA), when present in excess, was able to stimulate Ssq1 ATPase activity 3-fold (Fig. 4*C*). However, when Ssq1(V472F) was tested, no stimulation of ATPase activity was observed in the presence of Jac1(AAA). Thus, compensation of the low affinity of Ssq1(V472F) for substrate requires Jac1 with a fully functional J-domain. We also tested the affinity of Jac1 for Ssq1(V472F) by assessing its ability to stimulate ATPase activity in the absence of Isu1. When in 50-fold excess, Jac1 stimulated wild-type and mutant Ssq1 similarly, 4.1- and 3.9-fold, respectively. We conclude that the V472F alteration does not affect the affinity of Jac1 for Ssq1.

Up-regulation of Isu1 in ssq1(V472F)—Because the results of *in vitro* assays indicated that an increase in the concentration of Isu1 and Jac1 enhanced the ATPase stimulation of Ssq1(V472F), we asked whether such a mechanism might function *in vivo*. The levels of Jac1 and Isu1 were compared in extracts of mitochondria derived from wild-type and *ssq1(V472F)* cells. The amount of Jac1 in mitochondria from

these strains was similar (Fig. 4*D*). However, the levels of Isu1 were ~10-fold higher in both *ssq1(V472F)* and Δ*ssq1* cells compared with wild-type cells.

Synthetic Growth Phenotype of Cells Harboring ssq1(V472F) and Isu1 Mutations Encoding Alterations in the PVK Motif—Because biochemical analyses described above revealed enhancement of defects in ATPase stimulation when Ssq1(V472F) was combined with either Isu1(V135A) or Isu1(P134A) (Fig. 4*A*), we tested whether phenotypic enhancement would occur *in vivo* when these mutant proteins were present in the same cell. *ssq1(V472F)* has no growth defect (Figs. 1*A* and 5*A*). We tested the effect *in vivo* of combining either *isu1(P134A),* which causes slow growth, or *isu1(V135A)*, which causes no phenotypic effect (12), with *ssq1(V472F)*. Combination of either *isu1(P134A)* or *isu1(V135A)* with *ssq1(V472F)* resulted in inviability (Fig. 5*A*). The synthetic lethal interaction between *ssq1(V472F)* and *isu1(V135A)* is particularly striking because of the very mild phenotypic effects of the individual mutations. However, because purified Isu1(V135A), even at high concentrations, did not significantly stimulate Ssq1 ATPase in the presence of Ssq1(V472F) mutant (Fig. 4*A*), the *in vivo* and *in vitro* results correlate well.

DISCUSSION

The null phenotype of a *ssq1* mutant encoding a single amino acid alteration in the predicted substrate binding cleft of Ssq1 underscores the importance of substrate binding in the physiological function of this Hsp70. This result is not surprising, as interaction with substrate proteins is fundamental to molecular chaperone function (1, 2). However, the fact that a mutation causing greater than a 10-fold decrease in affinity for Isu substrate resulted in only mild phenotypic affects was surprising.

What is behind the capacity for this chaperone machinery to tolerate so well a decrease in affinity for its substrate? First, we think the fact that Ssq1 and Jac1 appear to have only a single substrate protein, Isu, has allowed evolution of a system that is very robust. If an Hsp70 has only a single, or very few, substrates the Hsp70-substrate interaction can be highly specific. This high degree of selectivity likely prevents unproductive interactions with other proteins in the substrate binding cleft. On the other hand, general Hsp70 chaperones such as mitochondrial Ssc1 and bacterial DnaK, which interact with a wide variety of exposed hydrophobic sequences of partially unfolded proteins, must have relatively low sequence selectivity (1, 2). Similar arguments can be made for the J-protein partner of Ssq1, Jac1, as no evidence exists for substrates other than Isu1.

Therefore, together the highly specific interactions of Jac1-Isu1 and Ssq1-Isu1 may explain the ability of this chaperone system to withstand a substantial decreased ability of Ssq1 to interact with its substrate. Moreover, very similar saturation curves obtained for the ability of Jac1 and Isu1 to stimulate the ATPase activity of Ssq1(V472F) indicate that the Jac1-Isu1 complex interacts with mutant Ssq1(V472F) as a single entity. Interaction as a complex would provide necessary orchestration of two critical interactions: Isu1 with the peptide binding domain and the J-domain of Jac1 with the ATPase domain. Such synchronization would likely increase the chance of productive substrate binding even when substrate affinity is reduced, as is the case with Ssq1(V472F).

Although we propose that mutant Ssq1(V472F) interacts predominantly with the Jac1-Isu1 complex, we do not mean to imply that normally this is the predominant mode of initiation of the Ssq1 ATPase cycle. Our analysis of interaction of wild-type Ssq1 with wild-type Isu1 indicates that independ-

ent interaction of Jac1 and substrate with Ssq1 can initiate ATP hydrolysis as Jac1 and Isu1 affinities for Ssq1, calculated from reverse titration experiments, differ by at least 4-fold. Indeed, taken together, our results support the idea that interactions among components of the Ssq1 system are highly flexible. Either Jac1 or Ssq1 could first interact with Isu1, depending on the relative rate of complex formation, binding affinities, and effective concentrations of the individual components. Flexibility of the timing of interactions among components of the Hsp70 chaperone system is not unique to the specialized Ssq1 chaperone system, as independent interactions of DnaK with protein substrate and DnaJ has been demonstrated (7). In the case of general Hsp70 systems, however, such flexibility of interaction might work against the robustness of the system, as it would increase chances for competition between different components able to interact with a particular Hsp70.

A second reason that the Fe-S cluster chaperone machinery can tolerate decreases in affinity for its substrate Isu1 is that a mechanism exists for up-regulation of Isu1. The higher concentration of Isu1 found in mitochondria of *ssq1(V472F)* may be sufficient to overcome the decreased affinity for Isu1 caused by the alteration in the peptide binding cleft. Such an explanation is supported by the *in vitro* data and bolsters the idea that the ATPase cycle is a rate-limiting step in the biogenesis of Fe-S-containing proteins and iron homeostasis. However, elucidation of the exact role of molecular chaperones in this process, be it insertion of a cluster in the Isu1 scaffold or transfer of the cluster to recipient proteins (33, 34), requires further analysis.

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