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Sequence-specific Interaction between Mitochondrial Fe-S Scaffold Protein Isu and Hsp70 Ssq1 Is Essential for Their *in Vivo* Function*

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Isu, the scaffold for assembly of Fe-S clusters in the yeast mitochondrial matrix, is a substrate protein for the Hsp70 Ssq1 and the J-protein Jac1 *in vitro*. As expected for an Hsp70-substrate interaction, the formation of a stable complex between Isu and Ssq1 requires Jac1 in the presence of ATP. Here we report that a conserved tripeptide, PVK, of Isu is critical for interaction with Ssq1 because amino acid substitutions in this tripeptide inhibit both the formation of the Isu-Ssq1 complex and the ability of Isu to stimulate the ATPase activity of Ssq1. These biochemical defects correlate well with the growth defects of cells expressing mutant Isu proteins. We conclude that the Ssq1-Isu substrate interaction is critical for Fe-S cluster biogenesis *in vivo*. The ability of Jac1 and mutant Isu proteins to cooperatively stimulate the ATPase activity of Ssq1 was also measured. Increasing the concentration of Jac1 and mutant Isu together but not individually partially overcame the effect of the reduced affinity of the Isu mutant proteins for Ssq1. These results, along with the observation that overexpression of Jac1 was able to suppress the growth defect of an *ISU* mutant, support the hypothesis that Isu is "targeted" to Ssq1 by Jac1, with a preformed Jac1-Isu complex interacting with Ssq1.

Hsp70 is a ubiquitous molecular chaperone that is well known for the ability to interact with a wide variety of partially folded proteins, although it functions in many different physiological processes (1–4). Ssq1, a yeast mitochondrial Hsp70, is an exception to this rule. Like Hsc66 of bacteria, Ssq1 plays a specialized role in Fe-S cluster biogenesis, a critical step in the maturation of numerous proteins critical for cellular metabolism (5). Ssq1/Hsc66 interacts with a substrate protein, Isu/IscU (6, 7), the scaffold on which an Fe-S cluster is built prior to transferring to a recipient apoprotein (8, 9). Like other Hsp70s, Ssq1 and Hsc66 do not work alone but in collaboration with a J-type co-chaperone, Jac1 and Hsc20, respectively (7, 10). Results of *in vivo* and *in organelle* studies support the idea that Ssq1 and Jac1 function in Fe-S cluster biogenesis. Reduction of the activity of either Ssq1 or its J-protein partner Jac1 results in a dramatic decrease in the activity of enzymes containing a Fe-S cluster, an increased accumulation of mitochondrial iron (11–13), and a decrease in the assembly of an Fe-S cluster on mitochondrial ferredoxin (14, 15).

Although the exact mechanism of Ssq1 function in the biogenesis of Fe-S clusters is unknown, evidence indicates that Ssq1 (and Hsc66) uses the same basic biochemical properties as other members of the Hsp70 family to bind short peptide segments (1, 5). This substrate interaction is regulated by ATP binding and hydrolysis and modulated by J-protein co-chaperones that stimulate the hydrolysis of ATP, thus increasing the stability of the Hsp70-substrate interaction. Both Ssq1 and Hsc66 and their respective co-chaperones Jac1 and Hsc20 interact independently with Isu/IscU. In addition, Isu/IscU and Jac1/Hsc20 cooperatively stimulate Ssq1/Hsc66 ATPase activity, a strong indication that Isu/IscU is a substrate for the chaperone systems (7, 16).

As expected of an Hsp70-J-protein-substrate interaction, Jac1 interacts with Isu independently of adenine nucleotide, although the interaction of Isu with Ssq1 is nucleotide-dependent (7). Typical of an Hsp70-substrate interaction, Isu binds stably to Ssq1 in the presence of ADP, whereas no direct interaction between Isu and Ssq1 has been detected *in vitro* in the presence of ATP. Jac1, however, facilitates the formation of a stable Ssq1-Isu complex in the presence of ATP, suggesting that under physiological conditions when ATP concentrations are typically high, Jac1 may "target" Isu for Ssq1 binding. Such targeting of a protein substrate by J-type co-chaperone has been proposed for other Hsp70-J-protein systems (17–21).

Although the basic biochemical characteristics of the Ssq1-Jac1-Isu interactions are similar to other Hsp70-J-protein-substrate interactions, the system is unusual in that Isu, as a folded protein, is a substrate for the chaperone system. Recent biochemical studies have identified LPPVK as a unique sequence within bacterial IscU, responsible for the specific interaction between IscU and Hsc66 (22, 23). Single amino acid alterations within this sequence had profound affects on the ability of Hsc66 to interact with IscU. Moreover, the LPPVK sequence is conserved among members of the IscU/Isu protein family in both bacteria and eukaryotes, including yeast and human, suggesting that it might function as a conserved recognition signal for Hsp70s involved in the biogenesis of Fe-S centers.

Although IscU/Isu proteins are clearly able to interact with their respective chaperone systems (6, 7, 16), the biological importance of these interactions has not been tested. In *E. coli*, in which these interactions were first observed, redundant systems of Fe-S cluster assembly exist (24). Thus, it may not be surprising that inactivation of the Hsc66-Hsc20-IscU system has rather modest effects on cell growth (25, 26). In contrast, in

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yeast, in which redundant systems do not exist, cells lacking Isu are inviable. In addition, inactivation of the Ssq1-Jac1 system in yeast has strong phenotypic effects, a strain deleted for *SSQ1* grows very poorly (27), and *JAC1* is essential in most genetic backgrounds (28, 29).

Because both Isu and the Ssq1-Jac1 chaperone system are critical, mutations that lead to disruption of Isu-Ssq1 interactions would be expected to have strong phenotypic effects, if, in fact, this interaction is important for *in vivo* functions. Because of the identification of LPPVK as a sequence required for the IscU-Hsc66 interaction (22, 23), we tested *ISU* mutants encoding single amino acid alterations of this sequence. We found a positive correlation between the ability of LPPVK mutant proteins to functionally interact with Ssq1-Jac1 *in vitro* and the ability of the proteins to support growth in the absence of wild-type Isu. Moreover, our results provide evidence that Jac1-dependent targeting of Isu for Ssq1 binding requires direct interactions between Jac1 and Ssq1 as well as specific recognition of the three C-terminal residues of the Isu LPPVK motif by Ssq1.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Media, and Chemicals—Two proteins of the *Saccharomyces cerevisiae* mitochondrial matrix, Isu and Isu2, are related to bacterial IscU (12, 30). Isu1 and Isu2 are more than 80% identical and carry out very similar if not identical functions. A double *isu1 isu2* deletion mutant is inviable, although the individual mutants are not; in particular, the *isu2* mutant grows nearly as well as wild-type cells. In this study the double mutant having deletions of both *ISU1* and *ISU2* is referred to as Δ*isu* and is isogenic to W303 (12). Mutations of *ISU1* were constructed by site-directed mutagenesis (QuikChange protocol, Stratagene), using wild-type *ISU1* (−330 to +755) cloned in pRS314 that has a *TRP1* marker (31) as a template. For overexpression studies, the mutants were subcloned to the *TRP1*-marked 2μ vector pRS424. Wild-type *JAC1* (−350 to +840) was subcloned to the 2μ vector pRS426 that carries the *URA3* marker.

Null mutations in *ISU1* were isolated by the following procedure. A mutagenized library of *ISU1* was created by cloning PCR-amplified DNA using *Taq* polymerase (Promega, Madison, WI) with 0.5 mM manganese sulfate added to the reaction mixture into pRS314. The library was transformed into Δ*isu* carrying a wild-type *ISU1* gene encoding a six-residue histidine tag on pRS316. Individual transformants were tested for the ability to grow in the absence of the wild-type copy of *ISU1* by patching them onto plates containing 5-fluoroorotic acid (5-FOA).[1] Extracts were made from transformants unable to grow on 5-FOA, and Western analysis was performed to determine whether the mutagenized *ISU1* expressed a stable, full-length protein product. The wild-type Isu migrated more slowly on SDS polyacrylamide gels because of the presence of the His tag. DNA was isolated from transformants expressing stable full-length protein using the MasterPure yeast DNA purification kit from Epicenter (Madison, WI). Plasmids, rescued by electroporating the DNA into *E. coli* (Bio-Rad), were transformed into yeast to verify the null phenotype. The sequence of candidate DNAs that retested as null mutants was determined (University of Wisconsin, Biotechnology Center, Madison, WI).

Yeast were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) or on synthetic media prepared as described (32). All chemicals, unless stated otherwise, were purchased from Sigma.

Purification of Proteins—Recombinant $Jac1_{His}$ (28), $Mge1_{His}$ and $Mdj1_{His}$ (33), $Ssq1_{His}$ and Isu_{His} wild-type, and mutant proteins (7) were purified as described previously. Protein concentrations, determined using the Bradford (Bio-Rad) assay system with 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 (31). Growth of such strains was indistinguishable from their wild-type derivatives in medium containing different carbon sources at different temperatures (data not shown).

Glycerol Gradient Centrifugation—Glycerol gradient centrifugation was carried out as described previously (7). Purified proteins (Isu, Jac1, and Ssq1) alone or in combinations were placed in reaction mixtures (70 μl) containing 2 mM ATP or 2 mM ADP and incubated for 10 min at 25 °C. Then, 70 μl of this mixture was loaded onto a 3-ml linear 15–35% (v/v) glycerol gradient prepared with 2 mM ATP or 2 mM ADP, as indicated, and centrifuged at 2 °C in a Beckman SW60 rotor for 28 h at 46,000 rpm. Fractions (130 μl each) were collected from the top of the tube, and their protein contents were analyzed by SDS-PAGE followed by silver staining. Plots representing quantification of protein content were obtained by densitometry analysis using Quantity One software (Bio-Rad).

Steady-state ATPase Activity of Ssq1—The release of radioactive inorganic phosphate from [γ-^{32}P]ATP was measured as described (7). Briefly, reaction mixtures contained Ssq1 and other proteins, when indicated, in buffer A (40 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 10 mM $MgCl_2$). Reactions were initiated by the addition of ATP (2 μCi, Dupont NEG-003H, 3000 Ci/mmol) to a final concentration of 118 μM. Incubation was carried out at 25 °C and terminated at the indicated time points by the removal of 20-μl aliquots to an Eppendorf tube containing 175 μl of 1 M perchloric acid and 1 mM sodium phosphate. After the addition of 20 mM ammonium molybdate (400 μl) and isopropyl acetate (400 μl), samples were vigorously mixed, and the phases were separated by a short centrifugation. An aliquot of the organic phase (150 μl) containing the radioactive orthophosphate-molybdate complex was removed, and radioactivity was determined by liquid scintillation counting. Control reactions lacking protein were included in all experiments.

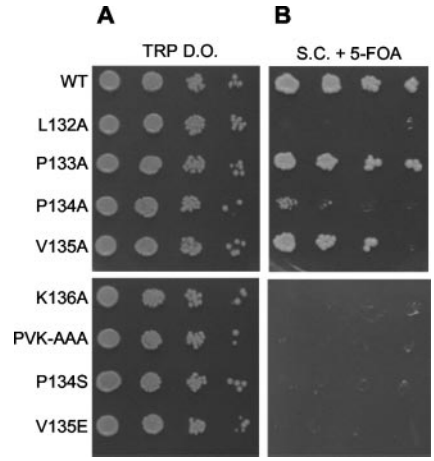

FIG. 1. **Mutations encoding amino acid substitutions in the LPPVK motif of Isu result in growth phenotypes.** Δ*isu* cells harboring plasmid-borne copies of both wild-type *ISU* (*URA3* marked), and mutant *ISU* (*TRP1* marked) were plated on glucose minimal medium lacking tryptophan (*TRP D.O.*) (*A*) or glucose minimal medium containing 5-FOA (*S.C. + 5-FOA*) (*B*). Plates were incubated at 30 °C for 3 days. 5-FOA selects for cells having lost the plasmid that has the wild-type copy of *ISU*.

RESULTS

Alterations in the LPPVK Motif of Isu Protein Affect Cell Growth—To study the biological importance of the LPPVK motif of Isu (residues 132–136), we constructed mutant genes encoding amino acid substitutions in this region, such that each of the residues was replaced individually with alanine. In addition, a mutant encoding a triple amino acid substitution, replacing the three C-terminal residues of LPPVK with alanines (PVK-AAA), was constructed. To test function, a Δ*isu* strain harboring a wild-type copy of the *ISU* gene and the *URA3* marker on a centromeric plasmid was transformed with plasmids carrying a second selectable marker and a mutant *ISU* gene. Growth of cells containing both wild-type and mutant copies of *ISU* were indistinguishable from that of cells harboring only a wild-type copy of *ISU* (Fig. 1*A*), indicating that none of the mutant *ISU* genes displayed a dominant-negative phenotype under the conditions tested.

To test the ability of the *ISU* mutants to support growth, the strains were plated on media containing 5-FOA. Only cells having lost the plasmid containing the *URA3* gene, and there-

[1] The abbreviation used is: 5-FOA, 5-fluoroorotic acid.

fore the wild-type copy of *ISU*, were able to survive on such media, thus allowing the growth phenotype of cells harboring only a mutant copy of *ISU* to be scored. Neither the triple mutant *Isu*(PVK-AAA) nor the single amino acid substitution mutants *Isu*(L132A) or *Isu*(K136A) was able to grow on 5-FOA medium (Fig. 1*B*). *Isu*(P134A) displayed a slow growth phenotype, whereas the *Isu*(P133A) and the *Isu*(V135A) mutants grew like the wild-type control.

To ensure that any growth defects observed were caused by altered protein function rather than the level of expression, mutant *ISU* protein levels were measured using cellular extracts prepared from cells expressing wild-type Isu tagged with six histidine residues in addition to a mutant Isu. Because His-tagged Isu migrates more slowly than the untagged protein on SDS polyacrylamide gels, a direct comparison of protein levels of wild-type with mutant Isu was possible. All mutant proteins were present at levels comparable with the wild-type Isu (data not shown).

Alterations in the LPPVK Motif Affect Isu Binding to Ssq1—To determine whether the growth phenotypes displayed by cells expressing mutant Isu proteins could be correlated with the biochemical properties of the proteins, we purified the mutant Isu proteins and tested their ability to bind Ssq1 using glycerol gradient centrifugation. Consistent with previous results (7), ~23% of wild-type Isu co-fractionated in the gradient with Ssq1 (fractions 13–17) in the presence of ADP, indicating the formation of a stable Isu-Ssq1 complex (Fig. 2). Mutant Isu(L132A) and Isu(P133A) showed similar patterns of co-migration, with approximately 35 and 16% of Isu protein, respectively, bound to Ssq1. In contrast, mutant Isu(P134A), Isu(V135A), and Isu(K136A), as well as Isu(PVK-AAA), migrated exclusively at a position characteristic of Isu alone (fractions 4–8), suggesting that the three residues Pro^{134}, Val^{135}, and Lys^{136} in the LPPVK motif might play key roles in forming a stable interaction with Ssq1.

To further address this question, we analyzed the interaction of the mutant Isu proteins in the presence of the J-protein co-chaperone Jac1 and ATP. Consistent with known Hsp70 biochemical properties, no stable interaction between Isu and Ssq1 was detected in the presence of ATP. As previously reported (7), however, the addition of Jac1 in the presence of ATP resulted in the formation of a stable Isu-Ssq1 complex that could be separated by glycerol gradient centrifugation from the Isu-Jac1 complex (Fig. 2). Under these conditions, ~52% of wild-type Isu co-localized with Ssq1 in fractions 12–17. The rest of Isu co-migrated with Jac1 in fractions 7–10, indicating the formation of a stable Isu-Jac1 complex. A similar pattern was observed for Isu(L132A) and Isu(P133A), with approximately 42 and 54%, respectively, of Isu co-migrating with Ssq1. Thus, mutant Isu proteins having a substitution of either of the first two residues of the LPPVK motif were able to form a stable complex with Ssq1 in the presence of either ADP or ATP and Jac1, just like wild-type protein.

A very different distribution of proteins was observed following glycerol gradient centrifugation of mutant proteins with changes in the last three residues (Isu(P134A), Isu(V135A), Isu(K136A), and Isu(PVK-AAA)) of the LPPVK motif (Fig. 2). In these cases only one peak, which co-localized with Jac1 protein, was observed, indicating formation of an Isu-Jac1 (fractions 7–10) but not an Isu-Ssq1 complex. Thus, consistent with the results obtained for the Isu-Ssq1 interaction in the presence of ADP, the formation of an Isu-Ssq1 complex in the presence of ATP and Jac1 was strictly dependent on the sequence of the last three amino acids in the LPPVK motif.

Alterations in the LPPVK Motif Affect the Ability of Isu to Stimulate Ssq1 ATPase Activity—As an alternative measure of

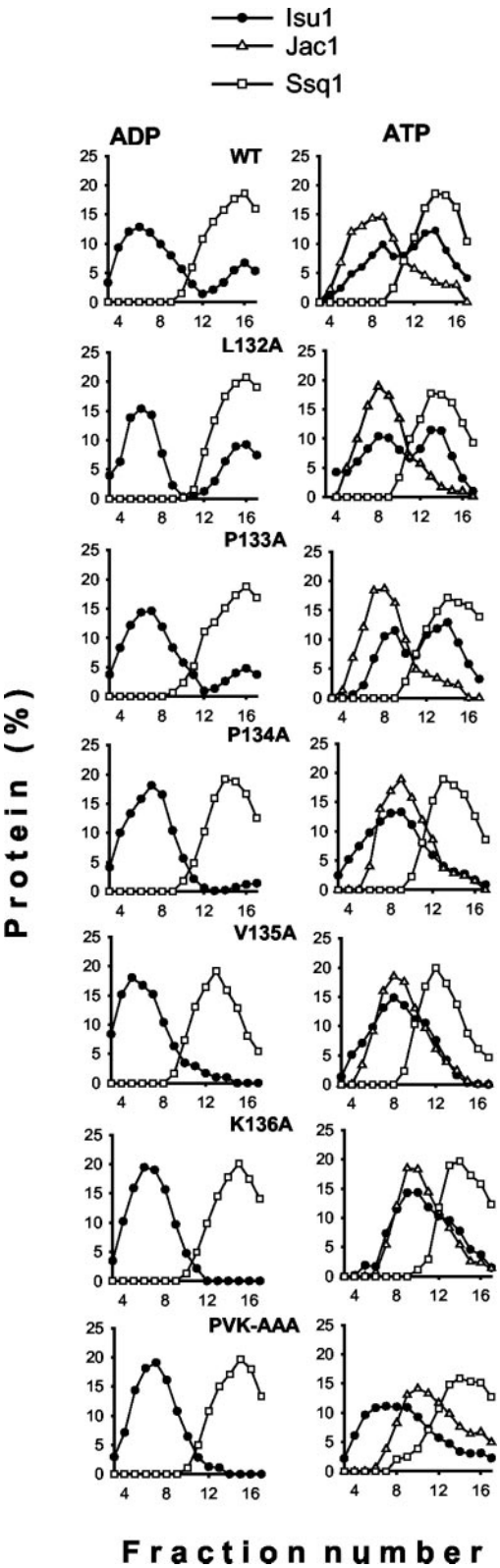

FIG. 2. **Amino acid substitutions within the LPPVK motif of Isu affect its interaction with Ssq1, both in the presence and absence of Jac1.** Isu binding to Ssq1 and Jac1 was analyzed using glycerol gradient centrifugation as described under "Experimental Procedures." Purified proteins, each at 5 μM concentration, were incubated in 70-μl reaction mixtures and loaded on 3 ml of 15–35% glycerol gradient. 2 mM ADP or ATP, as indicated, was present both in the reaction mixture and in the glycerol gradient. Plots representing quantification of protein content were obtained by densitometry analysis using Quantity One software (Bio-Rad) after analysis of protein content of fractions by SDS-PAGE and silver staining. *Filled circles*, Isu1; *open triangles*, Jac1; *open squares*, Ssq1.

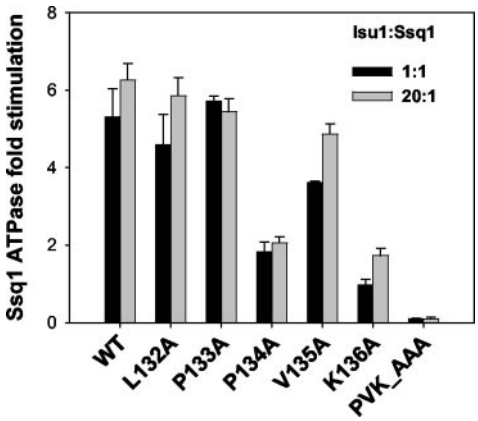

FIG. 3. **Stimulation of Ssq1 ATPase activity by Isu LPPVK mutant proteins.** Ssq1 ATPase activity was measured as described under "Experimental Procedures." The 80-μl incubation mixtures contained 0.8 μM Ssq1, 0.8 μM Jac1, 0.8 μM Mge1, and Isu at either 0.8 μM (1:1, *filled bars*) or 16 μM (1:20, *gray bars*) concentration in all cases, with the exception of Isu(L132A). Isu(L132A) protein was assayed at 0.8 and 8 μM.

Ssq1-Isu interaction, we tested the ability of the mutant Isu proteins to stimulate Ssq1 ATPase. As shown previously, efficient stimulation of Ssq1 ATPase activity requires the presence of the co-chaperone Jac1 and the nucleotide release factor Mge1 as well as the substrate protein Isu (7). Like wild-type Isu, Isu(L132A) and Isu(P133A) stimulated Ssq1 ATPase activity 5–6-fold under these conditions (Fig. 3). Moreover, increasing the mutant protein concentrations by 10–20-fold did not result in further stimulation of Ssq1 ATPase activity as was the case with wild-type protein. Thus, the ability of these mutant proteins to effectively stimulate ATPase activity was consistent with their ability to form stable complexes with Ssq1 (Fig. 2).

All alterations of the last three residues of LPPVK affected ATPase stimulation but not to the same extent. No stimulation was observed in the case of Isu(PVK-AAA), whereas Isu(K136A) and Isu(P134A) stimulated ATPase activity 1.5- and 2-fold, respectively (Fig. 3), consistent with their inability to support robust growth. On the other hand, Isu(V135A), which is able to support robust growth, stimulated ATPase activity 5-fold when present at high concentration, equivalent to wild-type stimulation.

Isu(P134S) and Isu(V135E) Are Strongly Impaired in Binding to Ssq1 As Well As in Stimulation of Ssq1 ATPase Activity—At first glance the behavior of the mutant proteins containing alanine substitutions for the last three residues of the LPPVK motif was consistent with a defect in binding Ssq1 because both their physical interaction with Ssq1 and their ability to stimulate Ssq1 ATPase activity were affected. The ability of Isu(V135A) to substantially stimulate Ssq1, however, albeit at a reduced level at least at low concentrations, was surprising because a stable complex with Ssq1 was not observed (Fig. 2). Yet, the substantial stimulation of Ssq1 ATPase by Isu(V135A) protein correlated well with the ability of Isu(V135A) to support rather robust cellular growth (Fig. 1). In addition, Isu(P134A), which showed a strong defect in both physical interaction and ATPase stimulation, could maintain viability of a Δ*isu* mutant, although growth was severely compromised.

To address these issues, we analyzed additional mutant genes Isu(P134S) and Isu(V135E) containing substitutions of Pro^{134} or Val^{135}. *Isu*(P134S) was isolated in a genetic screen selecting for null mutations in *ISU*. *Isu*(V135E) construction was based on the sequence homology between known Isu/IscU proteins and NifU from *Azotobacter vinelandii*, an Fe-S cluster scaffold protein that functions in the assembly of the nitrogenase protein (34). Although all known Isu homologues contain a valine at this position, NifU contains the sequence LPPEK. An analogous mutant of *E. coli iscU* was recently analyzed as well (23).

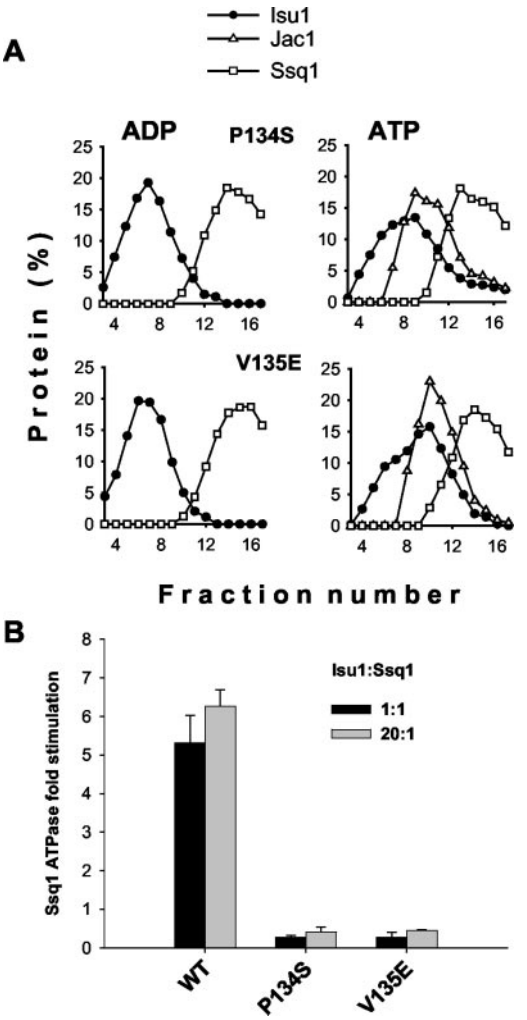

FIG. 4. **Isu(P134S) and (V135E) mutants are defective in Ssq1 binding and stimulation of Ssq1 ATPase activity.** *A*, glycerol gradient centrifugation was performed using experimental conditions described in Fig. 2*B*. The ATPase activity of Ssq1 was measured as described in Fig. 3. *Filled circles*, Isu1; *open triangles*, Jac1; *open squares*, Ssq1. *B*, Isu proteins were present at 0.8 μM or 16 μM concentrations with ratios of Ssq1:Jac1:Mge1:Isu of 1:1:1:1 (*filled bars*) and 1:1:1:20 (*gray bars*), respectively.

Neither *Isu*(P134S) nor *Isu*(V135E) was able to support cell growth (Fig. 1*B*). Moreover, neither of the mutant Isu proteins was able to form a stable complex with Ssq1, either in the presence of ADP or in the presence of ATP and Jac1 (Fig. 4*A*), but both mutant proteins were able to bind Jac1 as they comigrated with it during glycerol gradient centrifugation (Fig. 4*A*). Neither mutant protein was able to stimulate ATPase activity even when it was present at 20-fold higher concentrations than the other proteins in the ATPase assay. Thus, results obtained in both *in vivo* and *in vitro* experiments for these two additional *ISU* mutants substantiated the importance of the last three residues of the LPPVK motif for proper functioning of the Isu protein. Therefore, we propose that the biochemical properties of the Isu mutant proteins having alterations in the tripeptide PVK, the last three residues of the LPPVK motif, explain the defective growth phenotypes displayed by cells harboring these mutant alleles.

Presence of Jac1 Partially Compensates for the Lower Affinity of Isu Mutants for Ssq1—Stimulation of Ssq1 ATPase activity

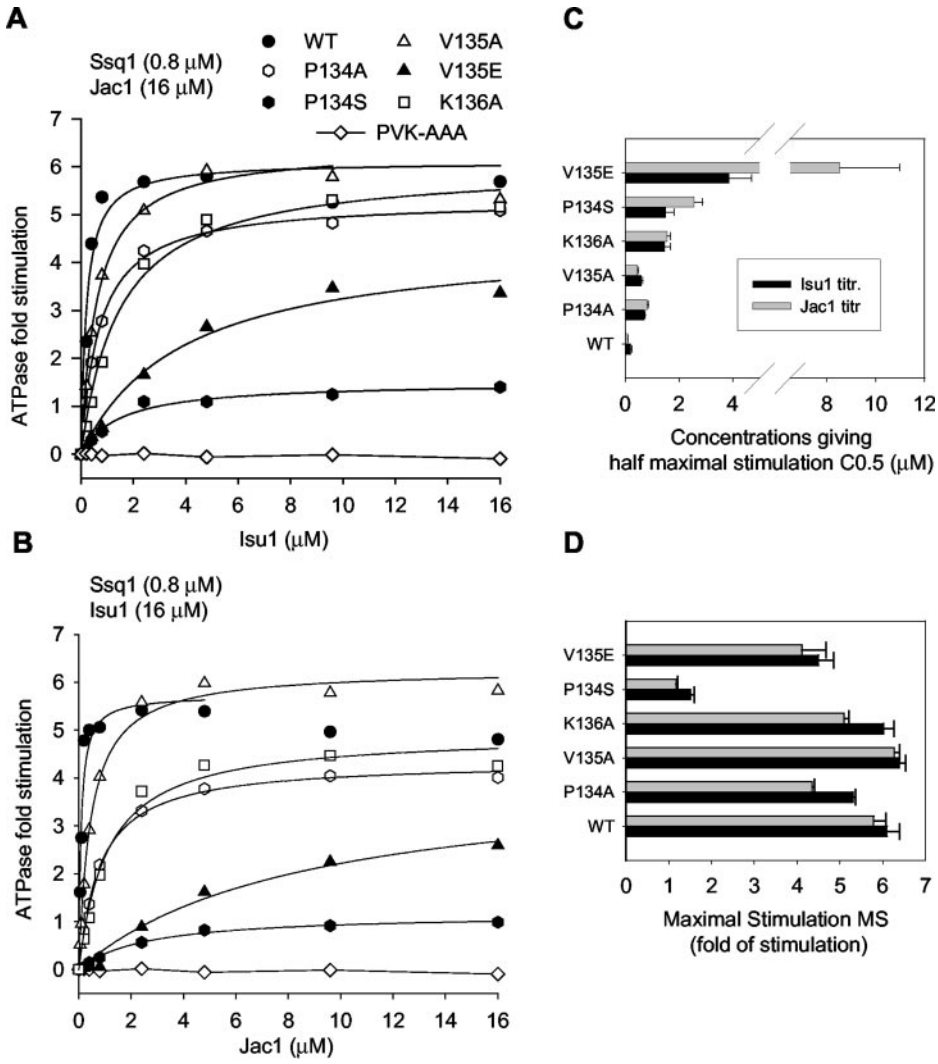

FIG. 5. **Stimulation of Ssq1 ATPase activity measured at different concentrations of Isu mutant proteins and Jac1 co-chaperone.** *A*, stimulation of Ssq1 ATPase was measured as described in Fig. 3 but with various concentrations of Isu mutant proteins. Concentrations of Ssq1 and Jac1 were as indicated. Mge1 was present at 0.8 μM in all reaction mixtures. *B*, concentrations of Jac1 were varied. *Curves* represent best fits to the data of Michaelis-Menten hyperbolic equation estimated by non-linear regression using Sigma-Plot software. *C*, concentration giving half-maximal stimulation ($C_{0.5}$) values calculated for Isu (*filled bars*) from plots presented in *A* and for Jac1 (*gray bars*) from plots presented in *B*. *D*, maximal stimulation (*MS*) values calculated for Isu from plots presented in *A* and for Jac1 from plots presented in *B*. Standard errors of estimated parameters are indicated. *Filled circle*, wild type (*WT*); *open hexagon*, P134A; *filled hexagon*, P134S; *open triangle*, V135A; *filled triangle*, V135E; *open square*, K136A; *open diamond*, PVK-AAA.

as well as stable binding of Isu to Ssq1 in the presence of ATP requires the presence of the co-chaperone Jac1. Because Jac1 forms a stable complex with Isu, it is quite possible that, similar to other known J-type co-chaperones, Jac1 is able to target substrate protein (Isu) for Ssq1 binding (7). To investigate whether Jac1 is able to compensate for the lower affinity of Isu mutants for Ssq1 binding, we examined the stimulation of Ssq1 ATPase activity in a concentration-dependent manner. Stimulation of Ssq1 ATPase activity was measured by titrating Isu wild-type and mutant proteins in the presence of excess Jac1 (Fig. 5*A*) or vice versa, by titrating Jac1 in the presence of an excess of either wild-type or mutant Isu (Fig. 5*B*). In all cases, a hyperbolic relationship between protein concentration and stimulation of ATPase activity was observed. Therefore, we were able to fit our data to the Michaelis-Menten equation, obtaining values for two parameters: the maximal stimulation and the concentration giving half-maximal stimulation ($C_{0.5}$). The values of the $C_{0.5}$ parameter can be taken as an approximate measure of Isu or Jac1 affinity for Ssq1. The maximal stimulation values have been interpreted as a measure of the efficiency of allosteric communication between the peptide binding domain and the ATPase domain of Hsp70 (23).

As seen in Fig. 5*D*, values for maximal stimulation obtained for most mutant proteins were within 75% of the wild-type value, regardless of whether Isu was titrated in the presence of excess of Jac1 or for Jac1 titrated in the presence of excess Isu. The only exception was Isu(P134S), which had a maximal stimulation of 25% of the wild-type value in both titrations. Thus, even though the mutant *ISU* proteins have a lower affinity for Ssq1, they are in most cases able to stimulate Ssq1 ATPase activity nearly as well as wild-type protein if Jac1 is also present at high concentrations.

Titration of mutant *ISU* proteins in the presence of excess Jac1 gave $C_{0.5}$ values significantly higher than the value obtained for wild-type Isu ($C_{0.5}$ = 0.21 μM), ranging from 3- to 18-fold higher, indicating that all mutant proteins tested had a lower affinity for Ssq1 (Fig. 5, *A–C*). Interestingly, a similar hierarchy was observed with titrations of Jac1 in the presence of excess Isu mutant proteins. This similarity is consistent with the idea that Jac1 and mutant Isu do not bind to Ssq1 independently but rather that first Jac1 interacts with Isu and then the Jac1-Isu complex interacts with Ssq1. If binding was independent, then one would expect that in each titration experiment the $C_{0.5}$ value obtained for Jac1 would be the same, regardless of which mutant Isu was present in excess.

Jac1 J-domain Mutant Binds Isu but Is Defective in Targeting Isu for Ssq1 Binding—To further characterize the role of Jac1 in the Isu-Ssq1 interaction, we tested whether Jac1 with an inactive J-domain was able to target Isu for Ssq1 binding. A Jac1 mutant protein in which the highly conserved HPD motif of the J-domain was replaced by three alanine residues, Jac1(AAA), was used. *jac1*(*AAA*) cells grew very poorly, indicating that a functional J-domain is essential for biological activity of Jac1 protein (28). As shown in Fig. 6*C*, Jac1(AAA) co-migrated with Isu during glycerol gradient centrifugation, indicating that alterations within the J-domain did not affect

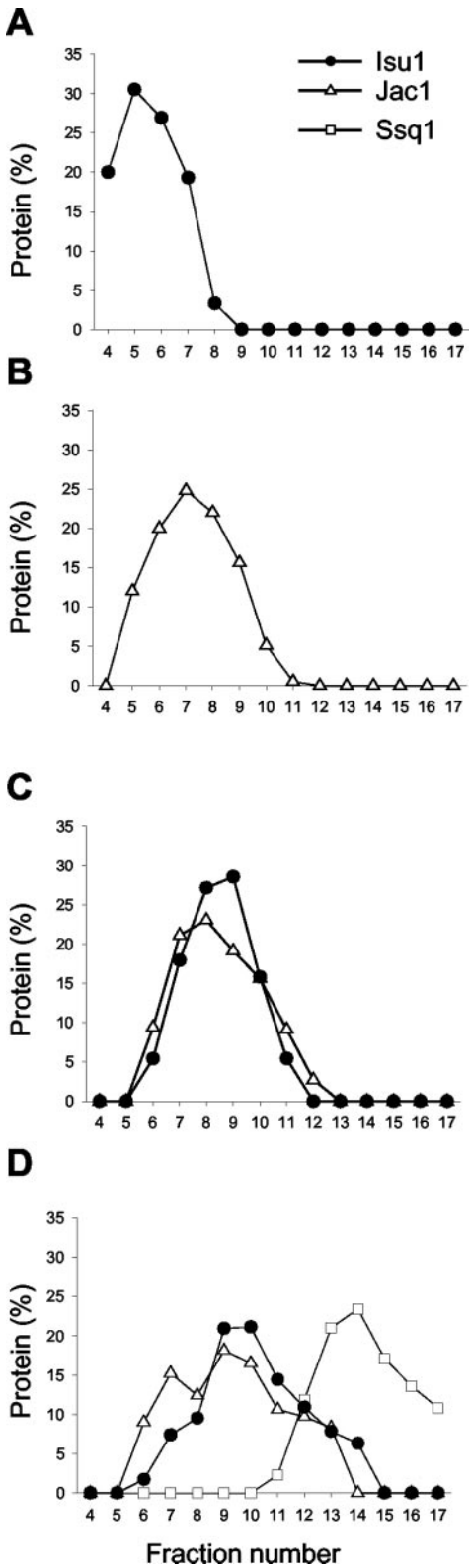

FIG. 6. **Jac1(AAA) mutant protein with altered J-domain HPD motif binds Isu but is defective in targeting Isu for Ssq1 binding.** *A*, glycerol gradient centrifugation was performed as described in Fig. 2*A*. Isu (5 μM). *B*, Jac1 (5 μM); *C*, Isu and Jac1 (5 μM each); *D*, Isu, Jac1, and Ssq1 (5 μM each). ATP at 2 mM was present in all reactions and gradients. *Filled circles*, Isu1; *open triangles*, Jac1; *open squares*, Ssq1.

interaction with Isu; however, Jac1(AAA) was not able to target Isu for Ssq1 binding effectively because no second peak of Isu was observed of Isu co-migrating with Ssq1 (Fig. 6*D*).

Next, we tested whether Jac1(AAA) was able to stimulate

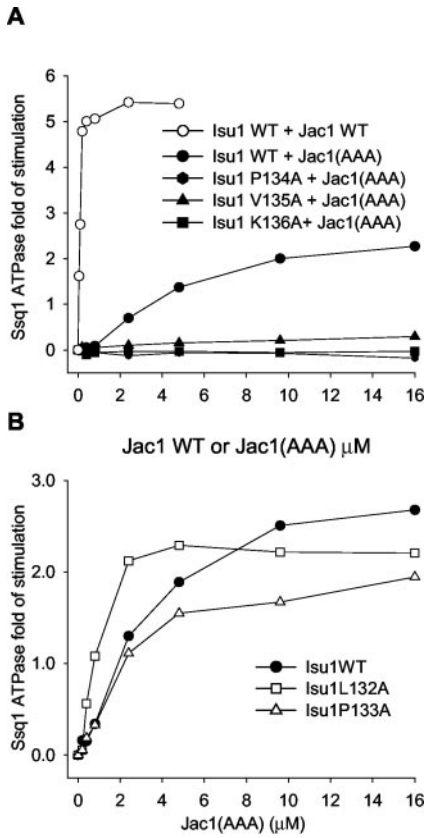

FIG. 7. **Isu mutant proteins defective in interaction with Ssq1 are unable to stimulate Ssq1 ATPase activity in the presence of Jac1(AAA) mutant.** *A*, the rate of ATP hydrolysis was measured as described under "Experimental Procedures." Reaction mixtures contained: 0.8 μM Ssq1, 0.8 μM Mge1, 16 μM Isu, and varying concentrations of Jac1. *Open circle*, Isu1 WT + Jac1 WT; *filled circle*, Isu1 WT + Jac1(AAA); *filled hexagon*, Isu1 P134A +Jac1(AAA); *filled triangle*, Isu1 V135A + Jac1(AAA); *filled square*, Isu1 K136A + Jac1(AAA). *B*, same procedure as described in *A*, but Isu wild-type and mutant proteins were at 8 μM concentrations. *Filled circle*, Isu1WT; *open square*, Isu1L132A; *open triangle*, Isu1P133A.

Ssq1 ATPase activity in the presence of wild-type and mutant Isu proteins. In the presence of wild-type Isu as well as in the presence of mutants Isu(L132A) and Isu(P133A) that bind Ssq1 normally Jac1(AAA) was able to weakly stimulate Ssq1 ATPase activity (2.5-fold *versus* the 6-fold observed for wild-type Jac1) (Fig. 7). In contrast, when Jac1(AAA) was incubated with Isu containing alterations of the last three residues of the LPPVK motif (Isu(P134A), Isu(V135A), and Isu(K136A)), no stimulation of Ssq1 ATPase activity was observed, further emphasizing the importance of both the J-domain of Jac1 and the last three residues of the LPPVK motif of Isu for promoting a functional interaction between Isu and Ssq1.

Overexpression of JAC1 Rescues the Growth Phenotype of Isu(P134A) Mutant—Cells harboring the Isu(P134A) mutant displayed a slow growth phenotype (Fig. 1), which correlated well with its reduced ability to functionally interact with the Ssq1-Jac1 chaperone system (Figs. 2 and 3). On the other hand, biochemical analyses indicated that increasing the Jac1 concentration could partially compensate for the reduced affinity of mutant Isu(P134A) for Ssq1, raising the question as to whether overexpression of Jac1 could improve the growth of an *Isu*(P134A) strain. To test this prediction, the Δ*isu* strain containing a copy of *Isu*(P134A) on a centromeric plasmid was transformed with a high copy plasmid harboring the *JAC1* gene (Fig. 8*A*). Indeed, overexpression of Jac1, which was confirmed by immunoblot analysis (data not shown), rescued the growth phenotype of *Isu*(P134A) cells both on rich media at

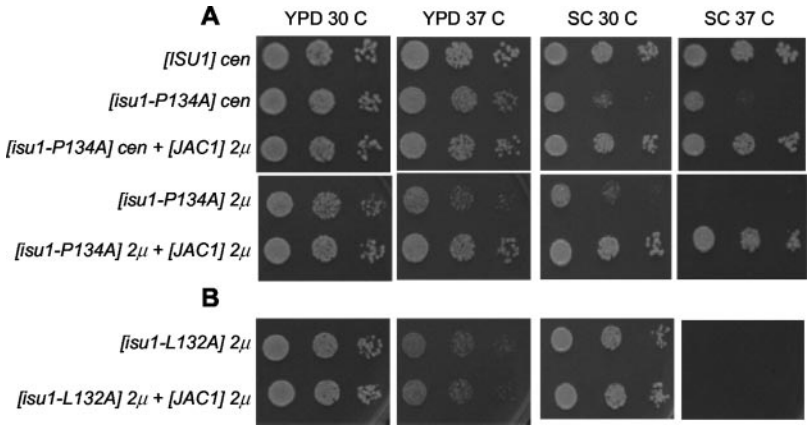

FIG. 8. **Overproduction of Jac1 and/or mutant Isu affects growth phenotype of cells harboring *ISU* mutants.** Δ*isu* cells harboring plasmids as indicated were plated on rich medium (*YPD*, 1% yeast extract, 2% peptone, and 2% glucose medium) and complete synthetic medium (*SC*) and grown for 3 days at indicated temperatures. *A*, overproduction of Jac1 suppresses growth phenotype of cells harboring *Isu*(P134A). *B*, overproduction of Isu(L132A) but not overproduction of Jac1 suppresses growth defect of cells harboring *Isu*(L132A).

37 °C and on minimal media at 30 and 37 °C. In contrast, overexpression of Isu(P134A) alone did not improve the growth rate. The latter result was consistent with the *in vitro* observation that even at high concentrations Isu(P134A) was not able to stimulate Ssq1 ATPase activity (Fig. 3) as long as Jac1 was present at a low concentration.

Inviablility of Isu(L132A) Is Rescued by Overexpression of the Mutant Protein—Isu(L132A) was the only mutant protein tested in which biochemical properties, formation of stable complex with Isu (Fig. 2), and efficient stimulation of Ssq1 ATPase activity (Fig. 3) were normal, but was unable to support cell growth (Fig. 1). To further investigate this apparent discrepancy, we examined how overproduction of Isu(L132A) affected cell growth. To our surprise, cells overexpressing Isu(L132A) were able to grow nearly as well as wild-type cells on both rich and minimal media at 30 °C (Fig. 8*B*). Suppression of the growth defect was not complete because these cells had a temperature-sensitive phenotype on both types of media. Overproduction of Jac1, however, did not improve growth of *Isu*(*L132A*) as anticipated because Isu(L132A) interacts normally with Ssq1 (Figs. 2, 3, and 7). We hypothesized that Isu(L132A) has a defect unrelated to its interaction with the Ssq1-Jac1 chaperone system.

DISCUSSION

Direct Interactions between Isu and Ssq1 Are Important in Vivo—Although it has been previously shown that the Isu scaffold protein and Ssq1 can physically interact (7, 35), the present study provides the first evidence that a direct interaction between the Fe-S cluster scaffold protein Isu and the molecular chaperone Ssq1 is critical *in vivo*. The three C-terminal residues of the LPPVK peptide of Isu are responsible for its functional interaction with Ssq1 because several amino acid substitutions in this motif inhibited both formation of the Isu-Ssq1 complex and the ability of Isu to stimulate Ssq1 ATPase activity. Most important, these biochemical defects correlate well with the growth phenotypes displayed by cells expressing these mutant Isu proteins. Proteins having a substitution of any one or all of these three residues by alanine as well as substitution of Pro^{134} by Ser and Val^{135} by Glu were unable to form a stable complex with Ssq1. With one exception, Isu(V135A), all were also defective in stimulation of Ssq1 ATPase activity. Consistent with these biochemical results, cells expressing all but Isu(V135A) were either inviable or grew extremely poorly. The efficient stimulation of the ATPase activity of Ssq1 by Isu(V135A) suggests that substitution of Val^{135} by Ala had a relatively weak effect on the functional interaction with Ssq1 and did not substantially affect *in vivo* function but that the effect was severe enough that a stable interaction was not maintained through overnight centrifugation. Interestingly, the recently reported structural characterization of *Thermotoga maritima* IscU leads to the prediction that the PVK tripeptide of Isu would be expected to be exposed on the surface in a loop between two α-helices, consistent with the ability of Isu to serve as a substrate for Ssq1 when folded in a functional state (36).

Replacement of the first two residues of the LPPVK motif by alanine did not affect the ability of Isu protein to interact functionally with Ssq1. Isu(L132A) and Isu(P133A) formed stable complexes with Ssq1. Both mutant proteins were also able to stimulate ATPase activity of Ssq1 even when the J-domain of Jac1 was compromised, but unlike *Isu(P133A)* cells, which grew like the wild-type under every condition tested, *Isu(L132A)* cells were inviable. We conclude that the defect of Isu(L132A) is not directly related to its interaction with Ssq1 or Jac1. It is possible that interaction with other components of the Fe-S cluster assembly pathways, such as the cysteine desulfurase Nfs1 or the yeast frataxin homologue Yfh1, or interactions with recipient proteins for Fe-S clusters are defective. On the other hand, it is possible that this protein has problems folding properly *in vivo*. Either explanation is consistent with the fact that, unlike the other mutant proteins we tested, overproduction of Isu(L132A) restored growth.

PVK Tripeptide Might Be a Universal Recognition Signal for Hsp70 Involved in Biogenesis of Fe-S Clusters—Interestingly PVK are the same three residues of the LPPVK motif found by Vickery and co-workers (23) to be the most critical for interaction of IscU with Hsc66. The contributions of individual residues, however, in the PVK sequence toward the efficiency of interaction between Isu/IscU and their respective Hsp70 partners are not equivalent in the two systems. For Isu, the strongest negative effects were observed for substitution of Lys^{136} (Lys^{103} in IscU), whereas for bacterial IscU, the strongest effects were observed for changes in residue Pro^{101} (Pro^{134} in Isu). For both Isu and IscU proteins, replacement of the Val residue (Isu in Val^{135} and IscU in Val^{102}) with alanine had weaker effects on binding to Ssq1/Hsc66 chaperones and on stimulation of their ATPase activities than replacement of the same residue by glutamic acid (23). Taken together, the evidence obtained for bacterial and mitochondrial proteins strongly suggests that the last three residues of the conserved PVK tripeptide constitute a universal signal recognized by Hsp70s involved in Fe-S centers biogenesis in the Isu homologues found in a wide range of organisms from bacteria to mammals.

Role of Jac1 Co-chaperone in Isu Binding to Ssq1—Taken together, our results support the hypothesis that targeting of Isu to Ssq1 requires synchronization of two events: direct interaction between the PVK tripeptide of Isu with the peptide binding cleft of Ssq1 and stimulation of Ssq1 ATPase activity initiated by the interaction of the J-domain of Jac1 with the

ATPase domain of Ssq1. This synchronization is very likely provided by the formation of a stable Isu-Jac1 complex, with both components interacting simultaneously with Ssq1.

This hypothesis is supported by several observations. First, the idea that Jac1-dependent targeting of Isu requires functional interactions between the J-domain of Jac1 and Ssq1 is supported by the fact that alterations in the conserved HPD tripeptide of the J-domain, which does not interfere with formation of Jac1-Isu complex, abolished stimulation of Ssq1 ATPase activity and formation of a stable Ssq1-Isu complex. These *in vitro* results correlate well with the *in vivo* observation that the growth of cells expressing the Jac1(HPD-AAA) mutant is extremely compromised (28). Such dependence on the J-domain of a J-protein for targeting of a substrate protein to an Hsp70 is consistent with data for the canonical Hsp70-J-protein pair DnaK-DnaJ (17).

In addition, a synchronized mode of interaction is supported by analysis of mutant proteins having alterations in the LPPVK motif, which have no effect on the Jac1-Isu interaction. Very similar saturation curves were obtained for the ability of Jac1 and mutant Isu to stimulate Ssq1 ATPase activity in reverse titration experiments, indicating that only a simultaneous increase of both Jac1 and mutant Isu was able to partially overcome the effect of the reduced affinity of Isu mutants for Ssq1. Consistent with this *in vitro* observation, overproduction of Jac1 resulted in significantly improved growth of Isu(P134A) strain, whereas overproduction of Isu(P134A) protein alone did not.

In summary, both the *in vivo* and *in vitro* results are consistent with the idea that Jac1 and Isu interact with Ssq1 simultaneously as a Jac1-Isu complex. Verification of the importance of the Jac1-Isu complex formation for Isu targeting, however, will require analysis of mutant proteins defective in this interaction.

Possible Role of Molecular Chaperones in Biogenesis of Fe-S Clusters—Results presented here pave the road for a molecular understanding of the function of molecular chaperones in the biogenesis of Fe-S clusters in mitochondria. We provide direct evidence that the sequence-specific interaction between Isu and the Ssq1-Jac1 chaperone system is critical *in vivo*. How might molecular chaperones function in the biogenesis of Fe-S clusters? Although it is possible that Ssq1-Jac1 plays a general chaperone role by protecting Isu against aggregation and inactivation, we consider it much more likely that they play a more direct mechanistic role in Fe-S cluster biogenesis. The Ssq1-Jac1 system could be involved in either the formation of the Fe-S cluster on the Isu scaffold protein or in the transfer of the Fe-S cluster from Isu onto recipient proteins. Results obtained recently by Lill and colleagues (15) suggest that the latter function is more likely. Depletion of either Ssq1 or Jac1 *in vivo* led to an increase in the amount of iron bound to Isu, whereas the amounts of iron associated with proteins that are the recipients of Fe-S clusters were reduced. It is possible that Ssq1-Jac1 participates directly in the transfer of Fe-S from Isu to recipient proteins. A role involving facilitating dissociation of the Isu dimer, perhaps facilitating cluster transfer, is similar to a role found for the *E. coli* Hsp70 DnaK in dissociation of dimers during DNA replication (37). On the other hand, direct interaction between Isu and the Ssq1-Jac1 system might facilitate dynamic formation and dissociation of the multiprotein Fe-S assembly machinery that contains not only the Ssq1-Isu complex but also other proteins involved in biogenesis of Fe-S centers such as the cysteine desulfurase Nfs1, the ferredoxin Yah1, the ferredoxin reductase Arh1, and the frataxin Yfh1 (5, 38). Testing of this appealing hypothesis will require reconstitution from purified components.

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