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Characterization of the Interaction between the J-protein Jac1p and the Scaffold for Fe-S Cluster Biogenesis, Isu1p*

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Jac1p is a conserved, specialized J-protein that functions with Hsp70 in Fe-S cluster biogenesis in mitochondria of the yeast *Saccharomyces cerevisiae.* Although Jac1p as well as its specialized Hsp70 partner, Ssq1p, binds directly to the Fe-S cluster scaffold protein Isu, the Jac1p-Isu1p interaction is not well understood. Here we report that a C-terminal fragment of Jac1p lacking its J-domain is sufficient for interaction with Isu1p, and amino acid alterations in this domain affect interaction with Isu1p but not Ssq1p. *In vivo*, such *JAC1* mutations had no obvious phenotypic effect. However, when present in combination with a mutation in *SSQ1* that causes an alteration in the substrate binding cleft, growth was significantly compromised. Wild type Jac1p and Isu1p cooperatively stimulate the ATPase activity of Ssq1p. Jac1p mutant protein is only slightly compromised in this regard. Our *in vivo* and *in vitro* results indicate that independent interaction of Jac1p and the Isu client protein with Hsp70 is sufficient for robust growth under standard laboratory conditions. However, our results also support the idea that Isu protein can be "targeted" to Ssq1p after forming a complex with Jac1p. We propose that Isu protein targeting may be particularly important when environmental conditions place high demands on Fe-S cluster biogenesis or in organisms lacking specialized Hsp70s for Fe-S cluster biogenesis.

Biogenesis of iron-sulfur (Fe-S) clusters, prosthetic groups required for the function of a variety of proteins involved in redox reactions, catalysis, and environmental sensing, is an intricate, highly conserved process. Eukaryotes utilize proteins homologous to those encoded in the bacterial iron-sulfur cluster assembly operon with Fe-S clusters being formed on a highly conserved scaffold protein, called Isu in *Saccharomyces cerevisiae,* and subsequently transferred to a recipient apoprotein (1–3). The specialized mitochondrial J-protein, Jac1p, and its Hsp70 partner, Ssq1p, are also crucial components of the yeast system with Isu protein being the only known substrate of this chaperone system (4). Similarly, in bacteria, IscU, the ortholog of Isu1p, is the only known substrate for the specialized chaperone pair, HscB (J-protein) and HscA (Hsp70) (5).

Although Jac1p is highly specialized, it shares many properties with other J-proteins. All contain a conserved ~70-amino acid sequence known as the J-domain, which is named for the canonical member of this group DnaJ from *Escherichia coli*. The universal function of J-domains is stimulation of the ATPase activity of Hsp70s, an activity that requires a conserved histidine:proline:aspartic acid (HPD) tripeptide and results in stabilization of an interaction between an Hsp70 and its client protein. Such activity is critical for Jac1p function, as alteration of HPD to three alanines (AAA) profoundly affects the ability of Jac1p to stimulate Ssq1p ATPase activity and to rescue the lethal effects of the absence of Jac1p *in vivo* (6, 7).

Such ability to stimulate the ATPase activity of an Hsp70 is critical because of the differential effects of ADP and ATP binding on client (substrate) protein interaction. When ATP is bound to an Hsp70, substrate protein binding and release occur very rapidly. Upon hydrolysis of ATP to ADP, Hsp70 undergoes a conformational change that slows release of the substrate protein (8). Under physiological conditions, ATP concentrations are high, and Hsp70 bound to ATP is the biologically relevant form, ensuring rapid interaction with substrate proteins. ATPase activity, which is critical for stabilization of the Hsp70-substrate interaction, is stimulated by both interaction of the client protein in the substrate binding cleft and J-protein interaction. In addition to their stimulatory role, some J-proteins (*e.g.* DnaJ and Jac1p) also bind substrate proteins directly, and thus it has been suggested that they might "target" them to Hsp70 (9–13). Such J-protein-dependent client protein targeting to Hsp70 has been demonstrated *in vitro* using purified components (9–13). However, the physiological importance of this mechanism for proper function of Hsp70 has not been tested.

Although it is clear that Isu1p is a substrate of the Ssq1p-Jac1p chaperone system, the exact function of the chaperones in the process of Fe-S cluster biogenesis is still under debate. Current evidence from *S. cerevisiae* suggests that the chaperones are important in transferring the iron-sulfur cluster from Isu1p to an aporecipient protein rather than assembling the cluster on Isu1p (14, 15). However, to understand the mechanism of chaperone involvement in Fe-S cluster biogenesis, it is crucial to understand how the chaperones interact with Isu1p and the importance of this interaction *in vivo*. It is established that Ssq1p interacts with a specific peptide sequence on Isu1p, proline, valine, lysine (PVK) (7, 16, 17), which is located on an exposed loop between two α-helices (18). However, very little is known about how Jac1p or its bacterial ortholog HscB interacts with the Isu/IscU protein scaffold. The structure of HscB, which has been determined by x-ray crystallography, consists of a 75-amino acid N-terminal J-domain and an 83-amino acid C-terminal domain composed of a three-helix bundle (19).

No experimental information was available concerning which residues of Jac1p are important for scaffold binding or whether such binding is important *in vivo*. Therefore, we set out to obtain a *JAC1* mutant encoding a protein defective in interaction with Isu1p and to determine the *in vivo* effect of the decreased interaction. We found that a conserved patch of residues in the C-terminal domain of Jac1p is directly

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involved in Isu1p binding, as replacement of several such residues by alanines resulted in reduced affinity for Isu1p. Surprisingly, under normal growth conditions this *JAC1* mutant did not display a growth phenotype. However, when combined with an *SSQ1* mutation causing reduced affinity of Ssq1p for Isu1p, growth was defective even at optimal temperatures, suggesting that Isu1p targeting by Jac1p can facilitate Ssq1p-Isu1p interaction.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Media, and Chemicals—Strains of *S. cerevisiae* used in this study were derived from PJ53, which is isogenic to *W303*: *trp1–1/trp1–1 ura3–1/ura3–1 leu2–3,112/leu2–3,112 his3–11,15/his3–11,15 ade2–1/ade2–1 can1–100/can1–100 GAL2$^+$/GAL2$^+$ met2-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2.* Strains designated as wild type are *trp1–1 ura3–1 leu2–3,112 his3–11,15 ade2–1 can1–100 GAL2$^+$ met2-Δ1 lys2-Δ2 jac1::ADE2* and contain pRS314-Jac. *JAC1* mutants were constructed by changing selected codons to encode alanine by site-directed mutagenesis (QuikChange protocol, Stratagene) using wild type *JAC1* (−350 to +824) cloned into pRS314 (20) as a template that has *TRP1* as a marker. In addition to the mutants discussed in the text, K125A/K129A, K132A/Q136A, C145A, K125A/K129A/K132A/Q136A, D120A/E121A, N147A/D148A, K162A/Y163A, L104A/K107A/D113A/Q117A, K125A/K129A/K132A/Q136A/L104A/K107A, K125A/K129A/K132A/K136A/D113A/Q117A, K162A/Y163A, D110A/D113A/E114A/Q117A, L104A/K107A/D110A/E114A, T98A/T99A/S100A, I135A/I147A, and D110A/D113A were constructed and analyzed. None of these mutants displayed a growth phenotype under the conditions tested. Plasmids containing mutant *JAC1* were transformed into the heterozygous diploid (*JAC1/jac1::ADE2*), and tetrads were analyzed for the desired progeny. For overexpression studies, *jac1*(*LKDDEQ*) and *JAC1* were subcloned to the *HIS3* marked 2μ vector pRS423 (21).

To assess the genetic interactions between *ssq1*(*V472F*) and *jac1*(*LKDDEQ*), the *ssq1*(*V472F*) strain was crossed to the Δ*jac1* + pRS316-Jac-His strain. Haploid *ssq1*(*V472F*) Δ*jac1* + pRS316-Jac-His progeny from this diploid were then crossed to *ssq1*(*V472F*) to yield a diploid *ssq1*(*V472F*)/*ssq1*(*V472F*) Δ*jac1*/*JAC1* + pRS316-Jac-His. Wild type *JAC1* and *jac1*(*LKDDEQ*) were transformed into this strain followed by sporulation to obtain the strains indicated in the text. To assess genetic interactions between *SSC1* and *jac1*(*LKDDEQ*), *JAC1* on 2μ vectors was transformed into Δ*ssq1*/*SSQ1* Δ*jac1*/*JAC1* and the desired strains obtained upon tetrad dissection. An empty *ADE2* vector was transformed into Δ*ssq1*/*SSQ1* to yield a Δ*ssq1* strain that was Ade^{2+} to ensure that any growth defects observed were not because of the presence of the *ade2* mutation in some strains. Yeast were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) or on synthetic media as described (22). All chemicals, unless stated otherwise, were purchased from Sigma.

Purification of Proteins—In all cases, protein concentrations, determined by using the Bradford (Bio-Rad) assay system with bovine serum albumin as a standard, are expressed as the concentration of monomers. Recombinant Mge1p$_{His}$ (23), Isu1p$_{His}$, and Ssq1p$_{His}$, the wild type and mutant proteins, were purified as described previously (4). To construct a plasmid for expression of Isu1p$_{Strep}$ having a Strep-tag (24) at the C terminus of the mature protein, a pET3a-Strep-tag vector was made by cloning the Strep-tag in as a linker using primers Strep-F 5′-gatcctggagccacccgcagttcgaaaaat-3′ and Strep-R 5′-gatcatttttcgaactgcgggtggctccag-3′, which allowed insertion of Strep-tag II (WSHPQFEK) into the BamHI site of pET3a, maintaining the 5′-BamHI but not the 3′-BamHI site. DNA encoding mature Isu1p (amino acids 37–165) was then cloned as an NdeI-BamHI fragment into the pET3a-Strep-tag to create pET3a-Isu-Strep-tag. To construct an expression vector for purification of the C terminus of Jac1p from *E. coli*, the C-terminal region from amino acids 71–184 with six histidine codons at the 3′-end were amplified by PCR to construct plasmid pET21d-Jac(71–184).

Expression of Isu1p$_{Strep}$ was induced in the *E. coli* strain C41 (25) by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at A_{600} = 0.6. After 3 h of growth at 30 °C, cells were harvested and lysed in a French press set to 15,000 p.s.i. in buffer L (20 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Triton X-100). The supernatant was loaded onto a Strep-Tacin column (IBA) equilibrated with buffer L. The column was washed with 15 column volumes of buffer L, eluted with buffer L with 2.5 mM desthiobiotin, and dialyzed against buffer K (20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 0.01% (v/v) Triton X-100, 200 mM KCl).

Jac1p$_{His}$ mutant proteins were purified according to the original protocol or by modifying the original protocol to a batch procedure (6). Proteins were eluted from a column with a 30–300 mM gradient of imidazole in buffer NI (20 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride) or in the case of the batch purification with a step elution in buffer E (20 mM Tris, pH 8.0, 10% (v/v) glycerol, 0.5 M NaCl, 200 mM imidazole). Fractions containing protein were then dialyzed to buffer B (20 mM Tris, pH 8.0, 10% (v/v) glycerol, 100 mM KCl).

Expression of the C terminus of Jac1p (Jac(71–184)) was induced in the *E. coli* strain C41 (25) by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at A_{600} = 0.6. After 3 h of growth at 30 °C, cell were harvested and lysed in a French press set to 16,000 p.s.i. After a clarifying spin, the supernatant was loaded on 2.5 ml of nickel-nitrilotriacetic acid-agarose at 4 °C, and after washing with buffer A (20 mM Tris-HCl, pH 8.0, 1 M $NaCl_2$, 2 mM $MgCl_2$, 1 mM ATP, 30 mM imidazole; 40 column volumes), protein was eluted by a 30–300 mM linear imidazole gradient in buffer NI (30 ml at 0.4 ml/min). Fractions containing Jac(71–184) were collected and dialyzed overnight in buffer B, then loaded on a Q-Sepharose (Amersham Biosciences) column equilibrated with buffer B. After washing with 10 volumes of buffer B, protein was eluted with linear gradient of 50–300 mM NaCl in buffer B (40 ml at 0.3 ml/min). Fractions containing Jac(71–184) were dialyzed for 4 h against buffer B, then loaded on a nickel-nitrilotriacetic acid-agarose column at 4 °C (0.5 ml equilibrated with buffer B). Protein was eluted with buffer B containing 500 mM imidazole. Protein was dialyzed against buffer B and stored at −70 °C.

Pulldown Experiments—Single concentration and titration pulldowns were performed by incubating indicated concentrations of Isu1p$_{Strep}$ and Jac1p$_{His}$ in 150 μl of buffer LP for 30 min at room temperature (20 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 125 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.05% (v/v) Triton X-100, 50 mM imidazole). Nickel-nitrilotriacetic acid-agarose beads were equilibrated with buffer LP and incubated with 0.1% bovine serum albumin. 20 μl of beads were added to each reaction and incubated at 4 °C for 1 h with rotation. The protein bound to the beads was washed five times with 500 μl of buffer LP. After the final wash, sample buffer was added to the reaction mixtures, and after a short spin all the supernatant was loaded on a SDS-polyacrylamide gel. The gel was stained using Coomassie Blue or Sypro-Ruby (Molecular Probes) and quantified by densitometry analysis. No differences in results were observed when experiments were performed in the presence and absence of dithiothreitol. All interaction assays were performed with apoIsu1p.

Surface Plasmon Resonance (SPR) Analysis—SPR[3] studies were carried out at 25 °C with a Biacore 2000 instrument (Piscataway, NJ). Pep-

[3] The abbreviations used are: SPR, surface plasmon resonance; MS, maximal stimulation; $C_{0.5}$, concentration giving half-maximal stimulation.

tide P-PVK (LSLPPVKLHC) was cross-linked to the surface of the sensor chip CM5 by thiol coupling as recommended by the manufacturer. Purified Isu1 protein 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)) with the running buffer at a flow rate of 10 μl/min. 60 μl of buffer R containing purified Jac1p and other components as indicated were used for injections.

Circular Dichroism (CD)—Measurements were performed on an Aviv 62A DS circular dichroism spectrometer from 194 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 8.0, 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).

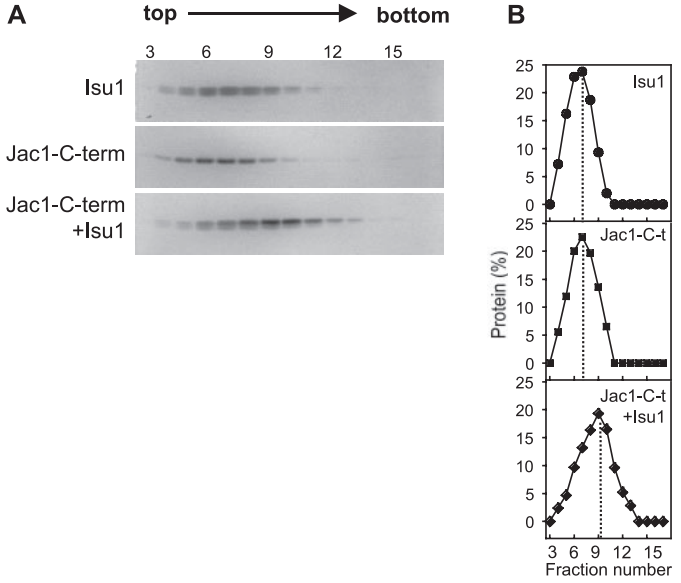

FIGURE 1. **The C-terminal domain of Jac1p binds Isu1p.** Binding of the C terminus of Jac1p to Isu1p was analyzed using glycerol gradient centrifugation as described under "Experimental Procedures." Purified proteins (5 μM) were incubated prior to loading on the gradient. *A*, fractions were collected from the top of the gradient and their protein contents assessed by SDS-PAGE followed by silver staining. *B*, plots representing quantification of protein content were obtained by densitometry analysis using Quantity One software (Bio-Rad).

Other Techniques—Steady state ATPase assays were carried out as described previously (4). 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. Glycerol gradient centrifugation was conducted as described in Ref. 4 but using 3 ml of 10–30% (v/v) glycerol gradient.

RESULTS

The C-terminal Domain of Jac1p Is Sufficient for Interaction with Isu1p—In addition to its J-domain, Jac1p contains a C-terminal 114-amino acid region. To test the prediction that the C-terminal region is sufficient for interaction with Isu1p, we purified a Jac1p truncation retaining amino acids 71–184 of the mature protein and assessed its ability to interact with Isu1p using glycerol gradient centrifugation and staining of the resulting fractions, a technique that has previously been used to analyze the interaction of Isu1p with full-length Jac1p (4). Upon centrifugation, the C terminus of Jac1p alone or Isu1p alone peaked in the seventh fraction. When mixed together prior to centrifugation, protein migrated further into the gradient, peaking in fraction 9, consistent with interaction of the Jac1p C terminus with Isu1p (Fig. 1). Because the two proteins co-migrate, immunoblot analysis was used to confirm a shift in migration of both Isu1p and Jac1p (data not shown). We conclude that the C terminus of Jac1p is sufficient for interaction with Isu1p.

Alterations in the C-terminal Domain of Jac1p Affect Isu1p Binding—To begin to identify the residues of the C terminus of Jac1p that are important for binding to Isu1p, alignments were made between the C terminus of Jac1p and HscB, the *E. coli* ortholog, the structure of which has been solved by x-ray crystallography (19). The 84-amino acid C termini, consisting of only the three α-helices, are 57% similar sharing 15 identical residues (Fig. 2*A*). We changed residues predicted to be on the surface of Jac1p to alanines, focusing on those conserved between HscB and Jac1p as well as charged residues, regardless of their conservation. In total, 22 residues were changed, initially in pairs (Fig. 2*A*, *bold residues*).

JAC1 is an essential gene. Therefore, to test the function of each mutant protein, a plasmid carrying a mutant *JAC1* was transformed individually into a heterozygous Δ*jac1/JAC1* diploid strain and the growth of the haploid progeny expressing only the mutant Jac1p that resulted from tetrad dissection analyzed. All *JAC1* mutants had a wild

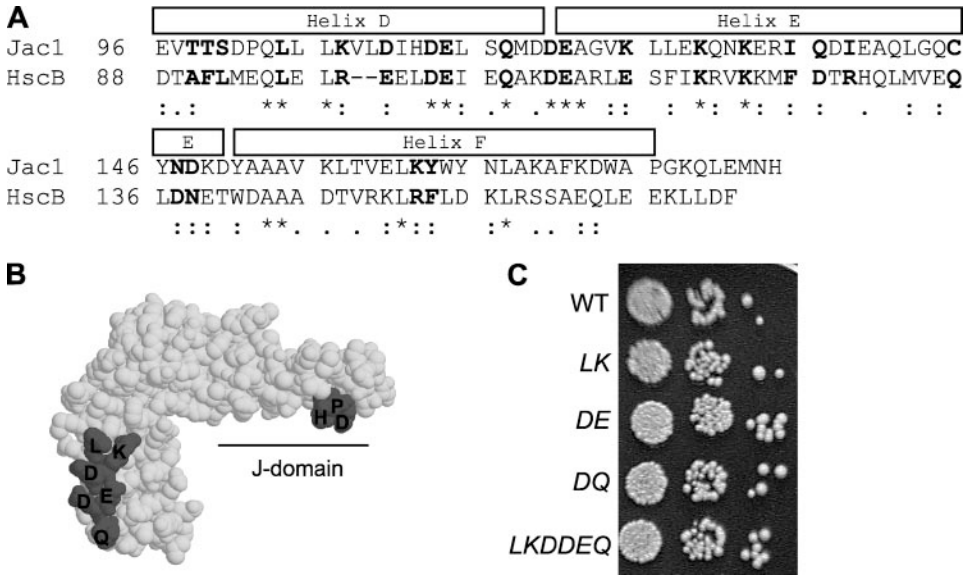

FIGURE 2. **Identification of a region of Jac1p important for binding Isu1p.** *A*, alignment of HscB and Jac1p C termini generated by SWISS-MODEL (33). Conserved residues, as determined by ClustalX (30), are indicated: identical (*), strong conservation (:), and weak similarity (.). Residues highlighted in *bold* indicate those changed to alanine. *B*, HscB structure (19) with residues corresponding to LKDDEQ and the HPD of the J-domain *highlighted*. The structure was prepared using Protein Explorer software. *C*, Δ*jac1* cells harboring plasmid-borne copies of wild type (*WT*) *JAC1* and mutant *JAC1* were plated on glucose-rich medium. Plates were incubated at 30 °C for 2 days.

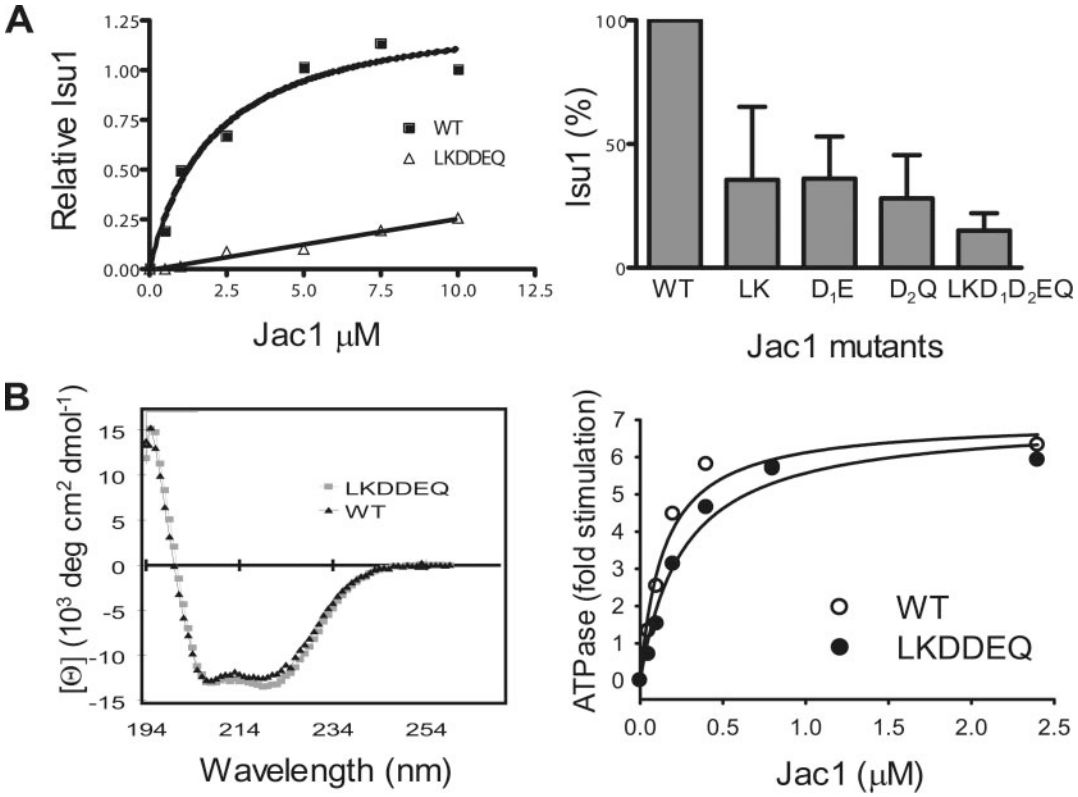

FIGURE 3. **Binding of LKDDEQ to Isu1p and stimulation of Ssq1p ATPase activity.** *A*, *left panel*, Isu1p (2.5 μM) and Jac1p wild type (*squares*) or LKDDEQ (*triangles*) with concentration as indicated was preincubated to allow complex formation. Nickel-nitrilotriacetic acid-agarose beads were added to pull down the complex. Bound Isu1p was quantitated by densitometry. The amount of Isu1p pulled down by 10 μM Jac1p wild type (*WT*) was set as 1. Values were plotted in Prism using a single binding hyperbola to fit data obtained for wild type Jac1p (K_d = 2.044 ± 0.969) and linear regression to fit Jac1p(LKDDEQ) data. *Right panel*, interaction of Isu1p (5 μM) and Jac1p wild type, LK, DE, DQ, or LKDDEQ (5 μM) was analyzed as described for the *left panel*. The amount of Isu1p interacting with Jac1p wild type was set to 100%. *Error bars* represent the standard deviation of six independent experiments. *B*, *left panel*, CD spectra measured for purified Jac1p wild type (*triangles*) and Jac1p(LKDDEQ) (*squares*) as described under "Experimental Procedures." *B*, *right panel*, Ssq1p ATPase activity was measured as described under "Experimental Procedures." Reaction mixtures contained 0.8 μM Ssq1p, 16 μM Isu1p, 0.8 μM Mge1p, and Jac1p wild type (*open circles*) or LKDDEQ (*filled circles*) as indicated. ATPase activity measured in the absence of Jac1p was set to 0. Evaluation of the data according to the Michaelis-Menten equation yielded the following parameters: wild type, maximal stimulation (MS) = 6.99 (±0.44), $C_{0.5}$ = 0.14 (±0.03); LKDDEQ, MS = 7.01 (±0.50), $C_{0.5}$ = 0.26 (±0.06).

type phenotype under a variety of growth conditions (Fig. 2*C*, data not shown). We then concentrated more thoroughly on a region of Jac1p predicted to have a cluster of conserved residues on the surface, L104A, K107A, D110A, D113A, E114A, Q117A (Fig. 2*B*), combining all six mutations in a single copy of the *JAC1* gene. *jac1*(*LKDDEQ*) had a wild type phenotype (Fig. 2*C*).

The lack of phenotypes of the mutants suggested to us that either the interaction with Isu1p was not disrupted or was not required *in vivo* under normal conditions. We then focused on the Jac1p(LKDDEQ) having 6-amino acid alterations. Wild type Jac1p and Jac1p(LKDDEQ), both having a His tag, were purified and their ability to interact with Isu1p compared. We first used glycerol gradient centrifugation. As the peaks representing Jac1p(LKDDEQ) and Isu1p did not shift when the two proteins were mixed (data not shown), we concluded that mutant Jac1p was defective in interacting with Isu1p. However, because of technical limitations of the centrifugation assay we were unable to test a variety of concentrations and thus assess the degree to which the affinity of the interaction was affected. Therefore, we developed a pulldown assay. Different concentrations of Jac1p were incubated with 2.5 μM Isu1p to allow complex formation. Nickel-nitrilotriacetic acid-agarose resin was then used to pull down Jac1p and any Isu1p bound to it, and protein was detected by staining after separation by SDS-PAGE. Binding of wild type Jac1p was saturable with an apparent K_d of ~2 μM (Fig. 3*A*, *left panel*). Jac1p(LKDDEQ) interacted with Isu1p less well than wild type protein, with only 25% as much binding as wild type observed at the highest concentration, 10 μM. Binding was not saturable at the concentrations tested.

To determine whether the observed defect could be attributed to a specific residue, assays were conducted using mutant *JAC1* proteins having combinations of two alterations within the Jac1p(LKDDEQ) motif. Equimolar concentrations of Jac1p and Isu1p were used. Jac1p(LKDDEQ) pulled down 20% as much Isu1p as wild type Jac1p (Fig. 3*A*, *right panel*). None of the mutant proteins containing two alterations were as defective in interacting with Isu1p as Jac1p(LKDDEQ), as all of the mutant proteins pulled down between 40 and 60% as much Isu1p as wild type (Fig. 3*A*, *right panel*). Because these results were consistent with the defect in interaction with Isu1p being attributable to at least 3-amino acid changes, we focused on Jac1p(LKDDEQ) in subsequent analyses.

Jac1p(LKDDEQ) Is Not Defective in Stimulation of Ssq1p ATPase Activity—The results described above indicate that Jac1p(LKDDEQ) is defective in interaction with Isu1p. To evaluate whether this decrease in Isu1p binding is a specific defect or because of general misfolding of the purified mutant protein, we carried out two experiments. First, CD spectra were obtained. The CD spectra of wild type Jac1p and Jac1p(LKDDEQ) were indistinguishable, indicating the differences observed in binding were not the result of global misfolding (Fig. 3*B*, *left panel*). Second, as a measure of the activity of the J-domains, we compared the ability of wild type Jac1p and Jac1p(LKDDEQ) to stimulate Ssq1p ATPase activity at a variety of concentrations in the presence of high concentrations of Isu1p. The degree of stimulation by

wild type and mutant Jac1p was very similar (Fig. 3*B*, *right panel*). Thus, by these two criteria Jac1p(LKDDEQ) appears to be folded properly, indicating that the defect in interaction with Isu1p is not because of global misfolding but rather because of the specific amino acid alterations in the C-terminal domain.

Interaction between Jac1p and Isu1p Is Required in Vivo if the Ssq1p-Isu1p Interaction Is Compromised—Jac1p(LKDDEQ) is defective in its interaction with Isu1p *in vitro* but is able to rescue a strain lacking Jac1p as well as the wild type protein, raising the possibility that the interaction between Jac1p and Isu1p is not required under normal conditions *in vivo*, perhaps because of the robust direct interaction between Ssq1p and Isu1p. To determine whether an interaction between Jac1p and Isu1p is critical when the Ssq1p-Isu1p interaction is compromised, we combined *jac1*(*LKDDEQ*) with a *SSQ1* mutant gene *ssq1*(*V472F*), which encodes an amino acid alteration in the peptide binding cleft. Ssq1p(V472F) has a greater than 10-fold reduction in affinity for Isu1p (26). *jac1*(*LKDDEQ*) and *ssq1*(*V472F*) cells grow indistinguishably from wild type cells (Fig. 4*A*). However, the double mutant *ssq1*(*V472F*) *jac1*(*LKDDEQ*) grows more slowly than either parent at the optimal growth temperature of 30 °C and is unable to form colonies at 37 °C. This growth defect is not because of a lower level of Jac1p(LKDDEQ) compared with wild type Jac1p, as immunoblot analysis of cell lysates indicated similar levels of Jac1p in the strains (Fig. 4*B*). In addition, a similar growth defect was observed even when the level of Jac1p(LKDDEQ) was ~8-fold higher than normal (data not shown).

Evidence of Targeting of Isu1p to Ssq1p by Jac1p in Vitro—This synthetic growth phenotype is consistent with the idea that the interaction of Jac1p with Isu1p is important when the direct interaction between Isu1p and Ssq1p is compromised and that such interaction facilitates formation of an Isu1p-Ssq1p complex. If this idea is correct, we would expect that the ability of Jac1p(LKDDEQ) to stimulate Ssq1p ATPase activity, when Isu1p is present, would be compromised, as an interaction of both Isu1p and Jac1p with Ssq1p is required for robust stimulation. Titration experiments were performed with Ssq1p(V472F). Isu1p was titrated in the presence of excess Jac1p or, in reverse, with an excess of Isu1p in varying concentrations of Jac1p. Because in both titration experiments, a hyperbolic relationship between protein concentration and stimulation of ATPase activity was observed, the data were fit to the Michaelis-Menten equation. This fitting allowed calculation of the protein concentration that yields half-maximal stimulation of the ATPase activity ($C_{0.5}$), a parameter that can be taken as an approximate measure of Isu1p or Jac1p affinity for Ssq1p. In the presence of Jac1p(LKDDEQ), the $C_{0.5}$ value is ~3-fold higher ($C_{0.5}$ = 3.45 μM, $C_{0.5}$ = 3.47 μM) than the value observed for wild type Jac1p titrating either Isu1p or Jac1p, respectively ($C_{0.5}$ = 0.99 μM, $C_{0.5}$ = 0.91 μM), indicating reduced affinity for both Isu1p and Jac1p(LKDDEQ). However, the similarity of the $C_{0.5}$ values determined for Isu1p and Jac1p(LKDDEQ) suggests that these proteins do not bind to Ssq1p(V472F) independently but rather that Jac1p(LKDDEQ) first interacts with Isu1p and then the Jac1p(LKDDEQ)-Isu1p complex interacts with Ssq1p(V472F), as is the case for wild type Jac1p and Isu1p interacting with Ssq1p(V472F) (Fig. 4*C* and Ref. 26). However, the 3-fold difference between the $C_{0.5}$ values for wild type Jac1p and Jac1p(LKDDEQ) is consistent with the fact that the ability of Jac1p(LKDDEQ) to bind Isu1p is compromised. Therefore, formation of Jac1p(LKDDEQ)-Isu1p complex requires high concentrations of both proteins and thus is a limited step in activation of Ssq1p(V472F) ATPase activity.

Altogether, results obtained for Jac1p(LKDDEQ) are consistent with our previous observation (26) that a high affinity interaction between Jac1p and Isu1p is necessary under certain cellular conditions. Although

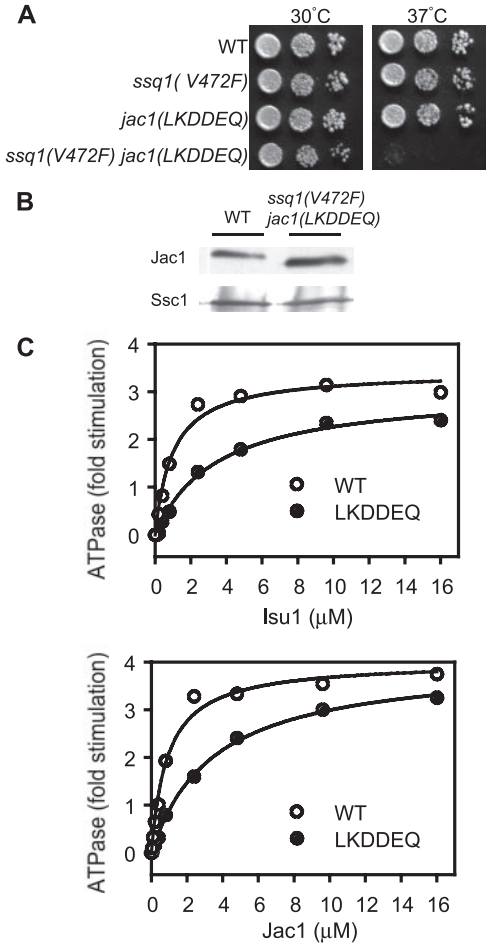

FIGURE 4. **Phenotypic effects of combination of *jac1(LKDDEQ)* with *ssq1(V472F)*.** *A*, Δ*jac1* cells harboring plasmid-borne copies of wild type (*WT*) *JAC1* and *jac1(LKDDEQ)* as well as Δ*jac1 ssq1(V472F)* cells harboring plasmid-borne copies of wild type *JAC1* and *jac1(LKDDEQ)* were plated on glucose minimal medium. Plates were incubated at 30 °C and 37 °C for 2 days. *B*, immunoblots of 0.1 optical density of cell lysates from indicated strains probed with polyclonal Jac1p antibody and Ssc1p antibody as a loading control. *C*, Ssq1p(V472F) ATPase activity was measured as described under "Experimental Procedures." *Top*, reaction mixtures contained 0.8 μM Ssq1p(V472F), 16 μM Jac1p wild type (*open circles*) or LKDDEQ (*filled circles*), 0.8 μM Mge1p, and various concentrations of Isu1p as indicated. ATPase activity measured in the absence of Isu1p was set to 0. Evaluation of the data according to the Michaelis-Menten equation yielded the following parameters: wild type, MS = 3.42 ± 0.15, $C_{0.5}$ = 0.99 ± 0.18; LKDDEQ, MS = 3.05 ± 0.14, $C_{0.5}$ = 3.45 ± 0.47. *Bottom*, reaction mixtures contained 0.8 μM Ssq1p(V472F), 16 μM Isu1p, 0.8 μM Mge1p, and Jac1p wild type (*open circles*) or LKDDEQ mutant (*filled circles*) as indicated. ATPase activity measured in the absence of Jac1p was set to 0. Evaluation of the data according to the Michaelis-Menten equation yielded the following parameters: wild type, MS = 4.01 ± 0.13, $C_{0.5}$ = 0.91 ± 0.12; LKDDEQ, MS = 4.03 ± 0.10, $C_{0.5}$ = 3.47 ± 0.24.

the phenotypic results suggest targeting of Isu1p to Ssq1p through a Jac1p-Isu1p complex can occur and under some circumstances be required, the reduction in ATPase stimulation we observed was quite small. The modest nature of the reduction may be because the reduction in affinity of Jac1p(LKDDEQ) for Isu1p compared with wild type Jac1p is less than an order of magnitude. To test more rigorously whether a high affinity interaction between Jac1p and Isu1p and hence targeting occurs *in vitro*, a substrate that interacted with Ssq1p but not Jac1p was required. We turned to a peptide derived from the amino acid sequence of Isu1p, P-PVK, containing the binding site of Ssq1p (26). First, the ability of Jac1p to interact with this peptide was examined using SPR. As expected, an interaction between wild type Jac1p and the P-PVK peptide was not observed (Fig. 5*A*). However, wild type Jac1p was capable of interacting with full-length Isu1p using this assay (Fig. 5*B*). Moreover, incubation of Jac1p with a 33-fold molar excess of P-PVK peptide did

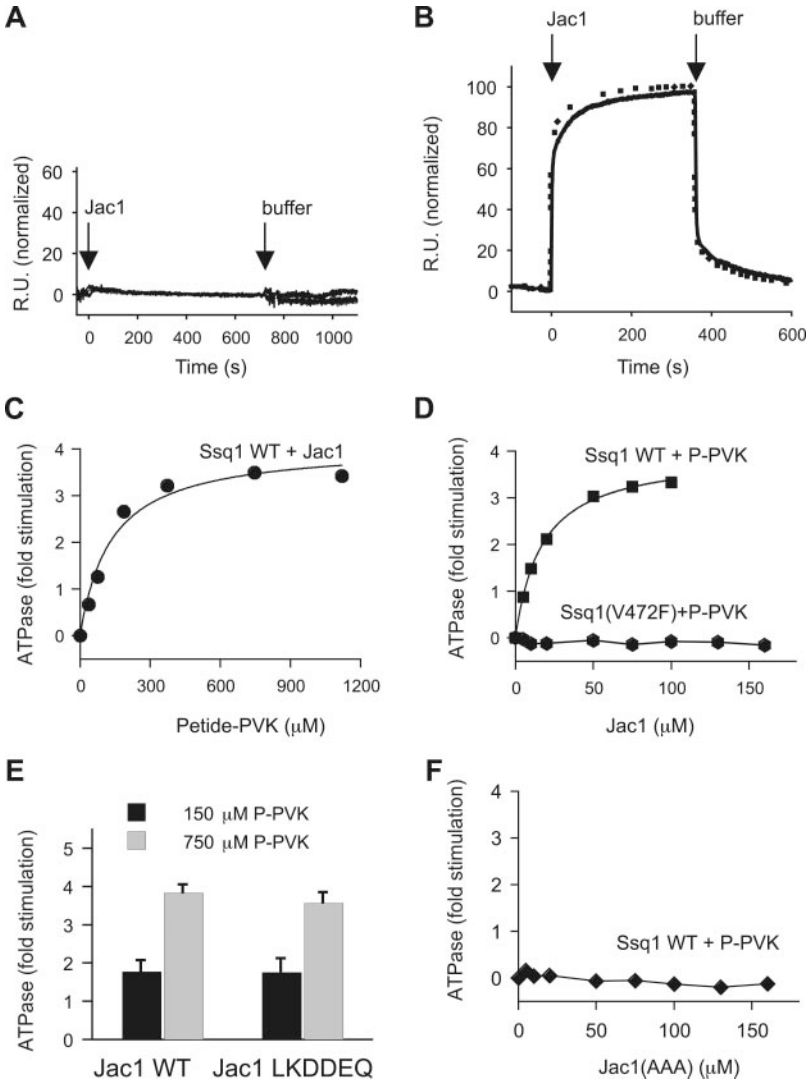

FIGURE 5. **PVK peptide, which does not bind to Jac1p, stimulates ATPase activity of Ssq1p.** *A*, SPR analysis of Jac1p binding to P-PVK peptide. Purified Jac1p at 2.5, 10, and 40 μM concentrations was passed over the chip surface with P-PVK peptide cross-linked to the surface of the chip (3000 response units) via its C-terminal cysteine residue. *B*, purified Isu1 protein was randomly cross-linked to the surface of SPR chip (2500 response units). Purified Jac1p at 9 μM was passed over the chip surface alone (*solid line*) or prior to injection was incubated for 10 min at room temperature in the presence of 300 μM peptide PVK (*dotted line*). *C*, Ssq1p ATPase activity was measured as described under "Experimental Procedures." Reaction mixtures contained 0.8 μM Ssq1p(wild type) (*WT*), 0.8 μM Mge1p, 75 μM Jac1p, and peptide P-PVK as indicated. ATPase activity measured in the absence of P-PVK was set to 0. Evaluation of the data according to the Michaelis-Menten equation yielded the following parameters: MS = 4.08 ± 0.25, $C_{0.5}$ = 132.9 ± 29.3. *D*, same as in *C*, but with 750 μM P-PVK and with Jac1p at various concentrations as indicated. Ssq1p(V472F) was at 0.8 μM concentration. ATPase activity measured in the absence of Jac1p was set to 0. Evaluation of the data according to the Michaelis-Menten equation yielded the following parameters: wild type, MS = 3.96 ± 0.06, $C_{0.5}$ = 16.9 ± 0.8. *E*, reaction mixtures contained 0.8 μM Ssq1p(wild type), 0.8 μM Mge1p, 75 μM Jac1p wild type, or LKDDEQ mutant as indicated and peptide P-PVK at 150 μM or 750 μM as indicated. ATPase activity measured in the absence of P-PVK was set to 0. *Bars* represent average values for four separate experiments. *Error bars* represent S.D. of measurements. *F*, same as in *D*, but with Jac1p(AAA) at concentrations as indicated.

not inhibit its ability to interact with immobilized Isu1 protein, indicating that Jac1p does not bind the P-PVK peptide. The P-PVK peptide provides a test condition in which Jac1p does not bind the Ssq1p substrate and, therefore, can be used to assess the importance of Jac1p-dependent substrate targeting *in vitro*.

First, we assessed whether Ssq1p ATPase activity was stimulated in the presence of Jac1p and P-PVK, even though Jac1p and the peptide do not interact. Titration of P-PVK in the presence of a high concentration of Jac1p (75 μM) resulted in maximal stimulation of Ssq1p ATPase activity of 4-fold (Fig. 5*C*), indicating that the efficiency of stimulation in the presence of P-PVK was lower than in the presence of high concentrations of full-length Isu1p (6-fold stimulation; Fig. 3*B*, *right panel*) and required a higher concentration of Jac1p ($C_{0.5}$ = 16.9 μM (Fig. 5*D*) *versus* $C_{0.5}$ = 0.14 μM in the presence of Isu1 protein (Fig. 3*B*, *right panel*)). A similar maximal stimulation of Ssq1p ATPase activity was observed for titration of Jac1p in the presence of 750 μM P-PVK (Fig. 5*D*). The $C_{0.5}$ values calculated for peptide P-PVK (132 ± 29.28 μM) and Jac1p (16.92 ± 3.96 μM) were 4.5-fold different, consistent with peptide and Jac1p interacting independently with Ssq1p.

Because these results indicate that independent interaction of Jac1p and Isu1p substrate can result in stimulation of Ssq1p ATPase activity, we next tested Ssq1p(V472F), which is defective in interacting with both Isu1p and the P-PVK peptide (26). In contrast to Jac1p(LKDDEQ) and Isu1p, no ATPase stimulation of Ssq1p(V472F) was observed using wild type Jac1p and peptide P-PVK, indicating that peptide P-PVK substrate interacting with Ssq1p(V472F) independently of Jac1p was unable to compensate for the lower affinity of Ssq1p for substrate (Fig. 5*D*). In addition, similar stimulation was observed using peptide P-PVK and wild type Jac1p or Jac1p(LKDDEQ), indicating that Jac1p(LKDDEQ) is not defective in interacting with Ssq1p (Fig. 5*E*). Furthermore, we also showed that independent interaction of P-PVK and Jac1p with Ssq1p is compromised when the J-domain of Jac1p is not active, as in the presence of Jac1p(AAA) and P-PVK no stimulation of the Ssq1p ATPase activity was observed (Fig. 5*F*). Together these results suggest that independent interaction of Jac1p and Isu1p substrate with Ssq1p is sensitive to reduction in the affinity of Ssq1p either for substrate or for its co-chaperone. Therefore, the defect observed with Ssq1p(V472F) and Jac1p(LKDDEQ) was a result of the decreased affinity of both Ssq1p(V472F) and Jac1p(LKDDEQ) for Isu1p (Fig. 4).

Binding of Jac1p to Isu1p Is Critical in the Absence of Ssq1p—Although Jac1p is an ortholog of HscB, Ssq1p appears to have resulted from a gene duplication during the evolution of the yeast lineage.[4] Most eukaryotes appear to utilize the multifunctional Hsp70 of the mitochon-

[4] B. Schilke, B. Williams, E. Craig, and J. Marszalek, unpublished results.

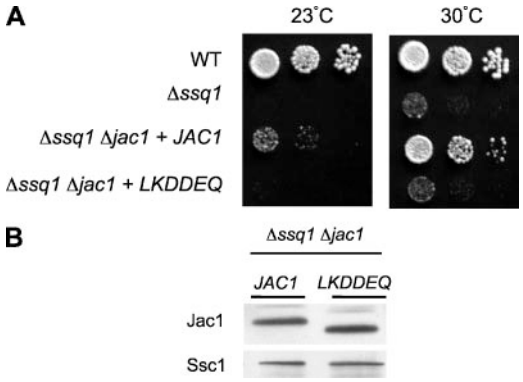

FIGURE 6. **Phenotypic effects of *jac1(LKDDEQ)* when Ssc1p functions in Fe-S cluster synthesis.** *A*, Δ*jac1* cells harboring plasmid-borne copies of wild type (*WT*) *JAC1* as well as Δ*ssq1* cells harboring plasmid-borne copies of *ADE2* and Δ*ssq1* Δ*jac1* cells harboring plasmid-borne *JAC1* or *jac1(LKDDEQ)* on high copy (2μ) plasmids were grown on glucose minimal medium lacking adenine at 23 °C for 4 days and at 30 °C for 3 days. *B*, immunoblots of 0.1 optical density of cell lysates from indicated strains probed with polyclonal Jac1p antibody and Ssc1p antibody as a loading control.

drial matrix that is also involved in general protein folding and translocation along with the specialized Jac1p in Fe-S cluster biogenesis. Consistent with this idea, cells lacking Ssq1p, although very compromised, are viable, and overexpression of either Ssc1p or Jac1p is capable of partially rescuing the growth (27, 28). Rescue of Δ*ssq1* cells by overexpression of Jac1 requires interaction with Ssc1p, as an alteration in the J-domain obviates the effect.[4] To test whether an Isu1p-Jac1p interaction is critical for rescue by Jac1p, we compared the ability of wild type Jac1p and Jac1p(LKDDEQ) to rescue Δ*ssq1*. Overexpression of *jac1*(*LKDDEQ*) did not improve growth even though the wild type and mutant proteins were overexpressed to the same level, ~8-fold over wild type levels (Fig. 6). This result suggests that an efficient interaction between Jac1p and Isu1p is required when Ssc1p is functioning in Fe-S cluster synthesis. Therefore, although the specialized system involving Ssq1p is capable of tolerating defects that decrease the affinity between Jac1p and Isu1p, the more general system is not.

DISCUSSION

The results presented here establish that the C-terminal domain of Jac1p is sufficient for interaction with Isu1p and identify residues in this region important for interaction with Isu1p. Both Hsp70s and J-proteins such as Sis1p and Ydj1p have hydrophobic binding clefts in which client proteins bind (29).[5] The lack of such a cleft on the triple helix bundle comprising the C terminus of HscB led to the prediction that a conserved acidic patch between residues 97 and 104 of HscB might be important for interaction with IscU (19). Our results support this idea, as four of the residues altered in Jac1p(LKDDEQ), which has a significantly reduced affinity for Isu1p, are located in the region of the protein predicted to be important for interaction. We were unable to identify specific residues of Jac1p critical for interaction with Isu1p. Alteration of the six residues in pairs had a modest effect on binding, suggesting to us that Jac1p and Isu1p interact over a broad surface area. Even the disruption of the six residues reduced the affinity less than 10-fold. This idea is also consistent with the failure of Vickery and colleagues (16) to identify a single peptide of IscU competent for HscB binding in their study that successfully identifies the PVK peptide sequence to which the Hsp70, HscA, binds.

The results presented here also indicate that the binding sites for Jac1p and Ssq1p on Isu1p are separable. Jac1p does not recognize the PVK motif as a binding site because a PVK-containing peptide derived from Isu1p does not bind to Jac1p, and mutations within the PVK motif of Isu1p do not affect formation of Jac1p-Isu1p complex (7). This result is reminiscent of the finding that DnaJ and its Hsp70 partner DnaK bind independently to their common substrate, RepA of bacteriophage P1 (31). Such independent binding likely facilitates targeting of Isu1p to Ssq1p. Therefore, on the one hand, the J-domain of Jac1p is responsible for stimulation of the ATPase activity, thereby promoting formation of Ssq1p-ADP and thus a high affinity for Isu1p; on the other hand, the transient interaction of C-terminal domain with Isu1p, independent from PVK motif, could serve to position Isu1p for interaction in the peptide binding cleft of Ssq1p. The rigidity of the structure of HscB (19) suggests that such positioning could be quite precise. In literature discussing the function of molecular chaperones, this process is referred to as substrate targeting. However, it can also be considered an example of a more general biochemical mechanism, important in regulation of many cellular processes including gene expression and signal transduction, referred to as recruitment (32). "Adhesive interactions" between two proteins bring one of the proteins physically close to its biological substrate (*e.g.* another protein and/or nucleotide sequence) thus imposing specificity. The residues involved in recruitment are typically well separated from those responsible for biological activity. In the case of interest here, Isu1p is recruited for productive interaction with Ssq1p by interacting with the "adhesive surface" of the C terminus of Jac1p.

So far, J-protein-dependent targeting, including recruitment of Isu1p by Jac1p, has been observed only *in vitro*, reconstituted with purified proteins (4, 9–11, 31). The identification of residues in Jac1p involved in binding Isu1p allowed us to examine the necessity of the interaction between Jac1p and Isu1p *in vivo*. Previously it has been shown that Jac1p can bind to Isu1p with this complex being targeted to Ssq1p or that Isu1p can bind to Ssq1p independently of Jac1p (7, 26). The results of our current study also support the idea of flexibility in binding order. A robust interaction between Jac1p and Isu1p does not appear to be important under typical laboratory conditions. However, if amino acid alterations that are introduced in Ssq1p reduce the affinity of Ssq1p for Isu1p, then an interaction between Jac1p and Isu1p becomes critical. The *in vitro* experiments conducted using P-PVK peptide derived from an Isu1p sequence illustrate that if the interaction between Jac1p and Isu1p was to be abolished *in vivo*, targeting would likely be essential.

The interaction between Jac1p and Isu1p is required when the general Hsp70, Ssc1p, rather than Ssq1p is functioning in iron-sulfur cluster biogenesis. It has recently been shown that Ssq1p is a specialized eukaryotic Hsp70 that exists only in certain fungi, with most eukaryotes having only a single multifunctional mitochondrial Hsp70.[4] However, because of the conservation of a Jac1 protein throughout the evolution, it is hypothesized that higher eukaryotic organisms lacking an Ssq1p homolog use the general mitochondrial Hsp70 in iron-sulfur cluster biogenesis. This then raises the interesting question of whether the interaction between Jac1p and Isu1p is critical even under optimal growth conditions in organisms lacking an Ssq1p homolog. The final answer to this question will require experiments with organisms containing only the general mitochondrial Hsp70. However, the fact that the ability of Ssc1p, the general mitochondrial Hsp70 of *S. cerevisiae*, to rescue a defect in Fe-S cluster biogenesis is more dependent on binding of Jac1p to Isu1p than is the function of Ssq1p (Fig. 6) supports this idea.[4] Because general Hsp70 must interact with many different client proteins as well as with different J-proteins responsible for processes such as protein import, protein folding, and Fe-S cluster biogenesis, selection of proper substrates may well be dependent on the targeting/recruitment mechanism provided by interaction of different J-proteins with

[5] Craig, E. A., Huang, P., Aron, R., and Andrew, A. (2006) *Rev. Physiol. Biochem. Pharmacol.* **156,** 1–21.

their specific substrate. Thus, one might predict that under such circumstances, compromised interaction between Jac1p and Isu1p would have devastating effects on cell functionality.

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